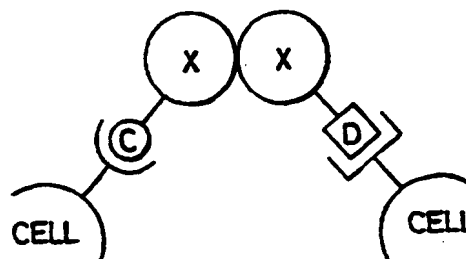
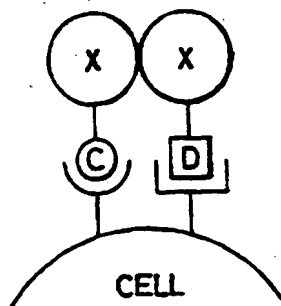
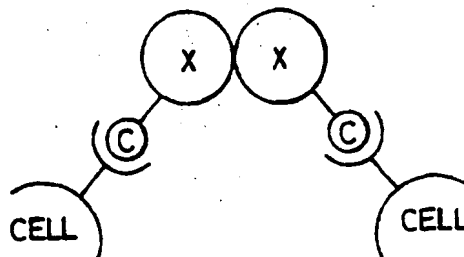
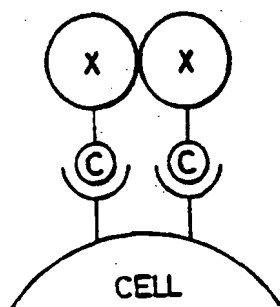




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(54) Title: COMPOUNDS FOR TARGETING



(57) Abstract

A compound comprising a target cell-specific portion and a directly or indirectly cytotoxic portion, characterised in that the target cell-specific portion recognises the target cell with high avidity. The cytotoxic portions of the compound may be a subunit of an oligomer, may contain a binding site for a small molecule, may be streptavidin, may be a dimeric ribonuclease or may be DNase-I. The target cell-specific portion may comprise two or more binding sites for the target cell, may be an antibody or fragment thereof, or may be an ScFv. A compound suitable for cell agglutination, particularly haemagglutination. A compound comprising a mediator portion and a directly or indirectly cytotoxic portion wherein the mediator portion recognises a target cell-specific molecule and provides a means of indirect targeting.

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COMPOUNDS FOR TARGETING

The present invention relates to compounds, some of which may be directly or indirectly cytotoxic combinations of compounds, that have a high avidity for, and can be targeted to, selected cells.

Background and Prior Art

The cell-specific targeting of compounds which are directly, or indirectly, cytotoxic has been proposed as a way to combat diseases such as cancer. Bagshawe and his co-workers have disclosed (Bagshawe (1987) *Br. J. Cancer* 56, 531; Bagshawe *et al* (1988) *Br. J. Cancer* 58, 700; WO 88/07378) conjugated compounds comprising an antibody or part thereof and an enzyme, the antibody being specific to tumour cell antigens and the enzyme acting to convert an innocuous pro-drug into a cytotoxic compound. The cytotoxic compounds were alkylating agents, eg a benzoic acid mustard released from para-N-bis(2-chloroethyl)aminobenzoyl glutamic acid by the action of *Pseudomonas sp.* CPG2 enzyme.

An alternative system using different pro-drugs has been disclosed (WO 91/11201) by Epenetos and co-workers. The cytotoxic compounds were cyanogenic monosaccharides or disaccharides, such as the plant compound amygdalin, which release cyanide upon the action of a β -glucosidase and hydroxynitrile lyase.

In a further alternative system, the use of antibody-enzyme conjugates containing the enzyme alkaline phosphatase in conjunction with the pro-drug etoposide 4'-phosphate or 7-(2'-aminoethyl phosphate)mitomycin or a combination thereof have been disclosed (EP 0 302 473; Senter *et al* (1988) *Proc. Natl. Acad. Sci. USA* 85, 4842).

Rybak and co-workers have disclosed (Rybak *et al* (1991) *J. Biol. Chem.* 266, 21202; WO 91/16069) the cytotoxic potential of a monomeric pancreatic ribonuclease when injected directly into *Xenopus* oocytes and the cytotoxic potential of monomeric RNase coupled to human transferrin or antibodies directed against the transferrin receptor. The monomeric RNase hybrid proteins were cytotoxic to human erythroleukaemia cells *in vitro*.

Other approaches are the *in vivo* application of streptavidin conjugated antibodies followed, after an appropriate period, by radioactive biotin (Hnatowich *et al* (1988) *J. Nucl. Med.* 29, 1428-1434), or injection of a biotinylated mAb followed by radioactive streptavidin (Paganelli *et al* (1990) *Int. J. Cancer* 45, 1184-1189). A pilot radioimmunolocalisation study in non-small cell lung carcinomas was conducted with encouraging results (Kalofonos *et al* (1990) *J. Nucl. Med.* 31, 1791-1796).

Apart from these examples, it is rather more common to see biotinylated antibodies and streptavidin-enzyme conjugates which are used in enzyme-linked immunosorbent assays.

These previous systems have used relatively large antibody-enzyme or antibody-streptavidin or antibody-biotin conjugates and may comprise portions of non-mammalian origin which are highly immunoreactive.

Rapid penetrance (Yokota *et al* (1992) *Cancer Res.* 52, 3402-3408) and rapid clearance (Colcher *et al* (1990) *J. Natl. Cancer Inst.* 82, 1191-1197) has been demonstrated for single chain Fv antibody fragments (ScFv).

In using the cell-specific reagents aforementioned in a therapeutically useful situation one of the requirements that needs to be met is for the

cell-specific reagent to accumulate to a sufficiently higher level at the target cell than at other cells. A further requirement is that a directly or indirectly cytotoxic reagent is carried to the target cell, and it is preferred that the said cytotoxic reagent is of high potency.

5

We have now devised improved systems at least some of which exhibit higher avidities to the selected target cells, and make use of novel, potent directly or indirectly cytotoxic agents.

10 Summary of Invention

A first aspect of the invention provides a compound comprising a target cell-specific portion and a cytotoxic portion characterised in that the cytotoxic portion has nucleolytic activity.

15

Suitably, as disclosed below, the cytotoxic portion may have ribonucleolytic activity or it may have DNA endonucleolytic activity.

20

One aspect of the present invention provides a compound comprising a target cell-specific portion and a directly or indirectly cytotoxic portion, characterised in that the target cell-specific portion recognises the target cell with high avidity.

25

A further aspect of the present invention provides a compound comprising a target cell-specific portion and a directly or indirectly cytotoxic portion characterised in that the cytotoxic portion is a sub-unit of an oligomer provided that, if the sub-unit is complexed with another sub-unit of the said oligomer then the said other sub-unit is the cytotoxic portion of a second compound of the invention.

30

A further aspect of the present invention provides a compound of at least two molecules each comprising a target cell-specific portion and a further portion wherein the molecules are complexed to one another via their further portions.

5

A further aspect of the present invention provides a compound comprising an oligomeric complex of at least two molecules each comprising a target cell-specific portion wherein the molecules are complexed to one another via their cytotoxic portions.

10

A further aspect of the present invention provides a compound comprising a target cell-specific portion and a directly or indirectly cytotoxic portion characterised in that the cytotoxic portion contains a binding site for a small-molecule wherein the said small-molecule binding site binds but does not modify catalytically the said small molecule.

15

A further aspect of the present invention provides a compound comprising a target cell-specific portion and a directly or indirectly cytotoxic portion characterised in that the target cell-specific portion comprises two or more binding sites for the target cell, wherein the target cell-specific portion is not an antibody, or bivalent fragment thereof, having respective arms which recognise the same entity as one another.

20

A further aspect of the present invention provides a compound comprising a target cell-specific portion and a cytotoxic portion characterised in that the cytotoxic portion has DNA endonucleolytic activity.

25

A further aspect of the invention provides a compound comprising a mediator portion and a directly or indirectly cytotoxic portion.

30

By "mediator portion" we mean the portion of the compound that recognises a target cell-specific molecule. The target cell-specific molecule may be a further compound of any of the appropriate preceding aspects of the present invention or it may be a target cell-specific molecule known in the art or it may be a derivative thereof capable of recognition by the mediator portion.

By "high avidity" we mean that the target cell-specific portion recognises the target cell with a binding constant of at least $K_d = 10^{-9}M$, suitably $K_d = 10^{-10}M$, more suitably $K_d = 10^{-11}M$, more suitably still $K_d = 10^{-12}M$, preferably $K_d = 10^{-13}M$, more preferably $K_d = 10^{-18}M$, more preferably still $K_d = 10^{-21}M$, yet even more preferably $K_d = 10^{-24}M$, and in further preference $K_d = 10^{-27}M$ or even $K_d = 10^{-30}M$.

By "target cell specific" portion we mean the portion of the compound which comprises one or more binding sites which recognise and bind to entities on the target cell. The said entities are expressed predominantly, and preferably exclusively, on the said target cell. The target cell specific portion may contain one or more binding sites for different entities expressed on the same target cell type, or one or more binding sites for different entities expressed on two or more different target cell types.

By a "directly cytotoxic agent" we mean an agent which in itself is toxic to the cell if it is to reach, and preferably enter the said cell.

25

By an "indirectly cytotoxic agent" we mean an agent which in itself may or may not be non-toxic, but which can bind specifically to a cytotoxic compound, or can bind specifically to a compound which can be converted into a cytotoxic compound by the action of a further reagent.

30

The entity which is recognised may be any suitable entity which is expressed by tumour cells, virally-infected cells, pathogenic microorganisms, cells introduced as part of gene therapy or even normal cells of the body which, for whatever reason, one wishes to target, but which is not expressed, or at least not with such frequency, in cells which one does not wish to target. The entity which is recognised will often be an antigen. Examples of antigens include those listed in Table 1 below. Monoclonal antibodies which will bind to many of these antigens are already known (for example those given in the Table) but in any case, with today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens. The antigen-specific portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "*Monoclonal Hybridoma Antibodies: Techniques and Applications*", J G R Hurrell (CRC Press, 1982).

The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* 81, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more

variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* 240, 1041); Fv molecules (Skerra *et al* (1988) *Science* 240, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* 242, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* 85, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* 341, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* 349, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

Chimaeric antibodies are discussed by Neuberger *et al* (1988, 8th *International Biotechnology Symposium* Part 2, 792-799).

Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into the framework of human antibodies.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments allows for rapid clearance, and may lead to improved tumour to non-tumour ratios. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and $F(ab')_2$ fragments are "bivalent". By "bivalent" we mean that the said antibodies and $F(ab')_2$ fragments have two antigen

combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining site.

Alternatively, the entity which is recognised may or may not be antigenic
5 but can be recognised and selectively bound to in some other way. For example, it may be a characteristic cell surface receptor such as the receptor for melanocyte-stimulating hormone (MSH) which is expressed in high number in melanoma cells. The cell-specific portion may then be a compound or part thereof which specifically binds to the entity in a non-
10 immune sense, for example as a substrate or analogue thereof for a cell-surface enzyme or as a messenger.

Preferably, the high avidity target cell specific portion comprises two or more different binding sites for the target cell.

15 The different binding sites for the target cell may or may not be two or more different antibodies, or fragments thereof, which are directed to different entities expressed on the target cell. Alternatively, the different binding sites for the target cell may recognise and selectively bind the cell
20 in some other, non-immune sense.

A further alternative is that one or more of the binding sites is an antibody, or part thereof, and that one or more of the binding sites for the target cell recognise and selectively bind the cell in some other, non-
25 immune sense.

A compound which has binding sites for two or more target cell-specific entities may be more specific for binding to the said target cell, and a compound which has more than one of each of the different binding sites
30 may bind to the said target cell with greater avidity. In combining two or

more binding sites, which in themselves may be of high specificity but low affinity, it will be possible to generate in the compound of the invention a higher affinity for the target cell whilst retaining the specificity of the binding sites.

Table 11. Tumour Associated Antigens

5	<u>Antigen</u>	<u>Antibody</u>	<u>Existing Uses</u>
	Carcino-embryonic Antigen	{C46 (Amersham) {85A12 (Unipath)	Imaging & Therapy of colon/rectum tumours.
	Placental Alkaline Phosphatase	H17E2 (ICRF, Travers & Bodmer)	Imaging & Therapy of testicular and ovarian cancers.
10	Pan Carcinoma	NR-LU-10 (NeoRx Corporation)	Imaging & Therapy of various carcinomas incl. small cell lung cancer.
	Polymorphic Epithelial Mucin (Human milk fat globule	HMFG1 (Taylor-Papadimitriou, ICRF)	Imaging & Therapy of ovarian cancer, pleural effusions.
15	Human milk mucin core protein	SM-3(IgG1) ¹	Diagnosis, Imaging & Therapy of breast cancer

- | | | |
|--|--------------------------|--|
| β -human Chorionic
Gonadotropin | W14 | Targeting of enzyme
(CPG2) to human
xenograft
choriocarcinoma in
nude mice. (Searle
<i>et al</i> (1981) <i>Br. J.</i>
<i>Cancer</i> 44 , 137-144) |
| A Carbohydrate on
Human Carcinomas | L6 (IgG2a) ² | Targeting of alkaline
phosphatase. (Senter
<i>et al</i> (1988) <i>Proc.</i>
<i>Natl. Acad. Sci. USA</i>
85 , 4842-4846) |
| 5 CD20 Antigen on B
Lymphoma (normal
and neoplastic) | 1F5 (IgG2a) ³ | Targeting of alkaline
phosphatase. (Senter
<i>et al</i> (1988) <i>Proc.</i>
<i>Natl. Acad. Sci. USA</i>
85 , 4842-4846) |

¹Burchell *et al* (1987) *Cancer Res.* **47**, 5476-5482

²Hellström *et al* (1986) *Cancer Res.* **46**, 3917-3923

10 ³Clarke *et al* (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1766-1770

Other antigens include alphafoetoprotein, Ca-125 and prostate specific antigen.

2. Immune Cell Antigens

5	Pan T Lymphocyte Surface Antigen (CD3)	OKT-3 (Ortho)	As anti-rejection therapy for kidney transplants.
	B-lymphocyte Surface Antigen (CD22)	RFB4 (Janossy, Royal Free Hospital)	Immunotoxin therapy of B cell lymphoma.
10	Pan T lymphocyte Surface Antigen (CD5)	H65 (Bodmer, Knowles ICRF, Licensed to Xoma Corp., USA)	Immunotoxin treatment of Acute Graft versus Host disease, Rheumatoid Arthritis.

3. Infectious Agent-Related Antigens

	Mumps virus-related	Anti-mumps polyclonal antibody	Antibody conjugated to Diphtheria toxin for treatment of mumps.
15	Hepatitis B Surface Antigen	Anti HBs Ag	Immunotoxin against Hepatoma.

It is preferable that the two portions of the compound of the invention are produced as a fusion compound by recombinant DNA techniques whereby a length of DNA comprises respective regions encoding the two portions of the compound of the invention either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired

properties of the compound. The benefits in making the compound of the invention using recombinant DNA techniques are several fold. Firstly, it enables a high degree of precision with which the two portions of the compound can be joined together. Secondly, the construction of compounds which are "hetero-oligomeric" can be controlled by the expression of the different recombinant DNA molecules encoding each of the different type of subunit of the "hetero-oligomer" in the same host cell.

By "hetero-oligomer" we mean those compounds in which two or more different cell-specific portions are joined to either the same or to different subunits which are capable of oligomerisation. The expression, in the same host cell of two compounds, A and B, each with different target cell specific portions but with a common second portion capable of oligomerisation will result in a mixed population of compounds. For example, if the common second portion is capable of dimerisation, three potential compounds will be produced: A_2 , AB and B_2 , in a ratio of 1:2:1, respectively.

The separation of the desired compound with each of the different cell specific portions, that is AB, can be achieved by two step affinity chromatography.

Application of the mixture of compounds to an affinity column specific for A will result in the binding of A_2 and AB. These compounds are eluted from this first column, and then applied to an affinity column specific for B. This will result in AB, but not A_2 , being bound to the column. Finally, the desired product AB, can be eluted.

Of course, the order in which the affinity columns are used is not

important.

The same principle of separating those compounds with two or more different binding sites can be applied to the purification of the desired
5 compounds from mixtures of other hetero-oligomers.

Conceivably, the two portions of the compound may overlap wholly or partly.

10 The DNA is then expressed in a suitable host to produce a polypeptide comprising the compound of the invention. Thus, the DNA encoding the polypeptide constituting the compound of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is
15 then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*,
20 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

25 The DNA encoding the polypeptide constituting the compound of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA
30 into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the
5 desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed
10 host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

15 Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

20

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

25

Those vectors that include a replicon such as a procaryotic replicon can also include an appropriate promoter such as a procaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

30

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites
5 for insertion of a DNA segment of the present invention.

Typical procaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and pTrc99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

10

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

15

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

20

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers
25 *his3*, *trp1*, *leu2* and *ura3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance,
30 complementary homopolymer tracts can be added to the DNA segment to

be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

- 5 Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini
10 with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

- The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large
15 molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme
20 and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

- Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International
25 Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* 239, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

Exemplary genera of yeast contemplated to be useful in the practice of the present invention are *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucosporidium*, *Botryosphaeria*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula*. Examples of *Saccharomyces* are *Saccharomyces cerevisiae*, *Saccharomyces italicus* and *Saccharomyces rouxii*. Examples of *Kluyveromyces* are *Kluyveromyces fragilis* and *Kluyveromyces lactis*. Examples of *Hansenula* are *Hansenula polymorpha*, *Hansenula anomala* and *Hansenula capsulata*. *Yarrowia lipolytica* is an example of a suitable *Yarrowia* species.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

Suitable promoters for *S. cerevisiae* include those associated with the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α -mating factor pheromone, α -mating factor pheromone, the *PRB1* promoter, the *GUT2* promoter, and hybrid promoters involving hybrids of parts of 5' regulatory regions with

parts of 5' regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

5 The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae*
10 *AHD1* gene is preferred.

The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell can be either procaryotic or eucaryotic. Bacterial cells are preferred
15 procaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eucaryotic host cells include yeast and
20 mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Preferred eucaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658 and monkey kidney-derived COS-1 cells available
25 from the ATCC as CRL 1650.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of
30 procaryotic host cells, see, for example, Cohen *et al*, *Proc. Natl. Acad.*

Sci. USA, 69: 2110 (1972); and Sambrook *et al*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989). Transformation of yeast cells is described in Sherman *et al*, *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY (1986). The method of Beggs, *Nature*, 275: 104-109 (1978) is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc, Gaithersburg, MD 20877, USA.

10

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern, *J. Mol. Biol.*, 98: 503 (1975) or Berent *et al*, *Biotech.*, 3: 208 (1985). Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

20

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

25

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of these cells, preferably a

30

monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium. Preferably, the culture also contains the protein.

- 5 Nutrient media useful for culturing transformed host cells are well known in the art and can be obtained from several commercial sources.

Alternatively, the target-cell specific and second portions of the compound of the invention are linked together by any of the conventional ways of
10 cross-linking polypeptides, such as those generally described in O'Sullivan *et al Anal. Biochem.* (1979) 100, 100-108. For example, the antibody portion may be enriched with thiol groups and the enzyme portion reacted with a bifunctional agent capable of reacting with those thiol groups, for example the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-
15 succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Amide and thioether bonds, for example achieved with m-maleimidobenzoyl-N-hydroxysuccinimide ester, are generally more stable *in vivo* than disulphide bonds.

- 20 Some of the various compounds of the invention are illustrated diagrammatically in Figure 1. C and D are the target cell-specific portions, and X is the cytotoxic portion. Of course, X may form higher order oligomers than those illustrated for example trimers, tetramers, pentamers, hexamers.

25

In Figure 1(a) to 1(d) C and D are shown binding to entities on either the same, or different cells.

- In one embodiment of the invention, C and D recognise different
30 molecules on the same target cell wherein the molecules on the same

target cell are not confined to that cell type but may occur on a few other cell types. In particular, C may recognise molecules on cell types I, II and III, whereas D may recognise molecules on cell types I, IV and V. Thus a compound of the invention comprising C and D as the target cell-specific portion will have greater specificity for cell type I compared with cell types II, III and IV. This aspect of the invention is particularly helpful, as there have been very few completely target cell-specific molecules discovered, whereas molecules which occur on a few cell types, and which are useful in this aspect of the invention, are well known.

Such molecules are usually cell-surface antigens for which cross-reactive antibodies are known. Examples of such molecules are given in Table 2.

Table 2

<u>Antigen</u>	<u>Cell-type</u>	<u>Antibody</u>
CD9	Pre-B cells, monocytes, platelets	MM2/57 (IgG2b, mouse)
CALLA	Lymphoid progenitor cells, granulocytes	B-E3 (IgG2a, mouse)
CD13	Myeloid monocytes, granulocytes	B-F10 (IgG1, mouse)
CD24	B-cells, granulocytes	ALB-9 (IgG1, mouse)
CD61	Platelets, megakaryocytes	PM 6/13 (IgG1, mouse)

The antibodies described in Table 2 are generally available from Serotec, Oxford, OX5 1BR, UK.

Preferably, the cytotoxic portion of the compound of the invention is capable of oligomerisation. Attachment of the target-cell specific portion to a cytotoxic portion capable of oligomerisation provides a method for increasing the number of binding sites to the target cell. For example, if the target cell-specific portion is joined to a portion capable of forming a dimer then the number of target cell-specific binding sites is two; if the target cell-specific portion is joined to a portion capable of forming a tetramer then the number of target cell-specific binding sites is four. The number of target cell-specific binding sites is greater than one and the compounds may therefore have a greater avidity for the target cell than do compounds which only have one target cell-specific binding site.

It is preferable for the cytotoxic portion of the compound of the invention capable of oligomerisation to contain no interchain disulphide bonds nor intrachain disulphide bonds; to be well characterised; to be non-toxic; to be stable; to be amenable to preparation in a form suitable for pre-clinical or clinical use or be in pre-clinical or clinical use; and for the subunit monomers to have a high affinity for each other, that is they contain one or more subunit binding sites.

20

Preferably, each subunit of the cytotoxic portion of the compound of the invention contains a binding site for a small molecule, the small molecule being capable of being conjugated to any from the following compounds: radioactive compound; spin-labelled compound; drug; pro-drug; radionuclide; protein including enzyme; antibody; or toxin.

25

In a preferred embodiment of the invention, the cytotoxic portion is streptavidin. Streptavidin is a homotetrameric molecule of $M_r = 60000$ (subunit $M_r = 15000$) and is produced by *Streptomyces*. Streptavidin binds four molecules of the water-soluble vitamin biotin with high

30

specificity and affinity ($K_d = 10^{-15}M$) although isolated subunits possess a very much lower affinity for biotin ($K_d = 10^{-8}M$). Each subunit of streptavidin has a tightly-packed "core", with relatively unstructured amino- and carboxyl-terminal extensions. These extensions are believed to contribute to the formation of higher order aggregates of streptavidin. Many commercial forms of streptavidin are extensively proteolysed, have lost their unstructured extensions, and form stable tetramers (Bayer *et al* (1989) *Biochem J.* 259, 369-376; Bayer *et al* (1990) *Methods Enzymol.* 184, 51-67). The mature form of the protein has been the subject of recent research and is becoming increasingly well characterised (Gitlin *et al* (1988) *Biochem J.* 256, 279-282; Gitlin *et al* (1990) *Biochem J.* 269, 527-530; Sano & Cantor (1990) *J. Biol. Chem.* 265, 3369-3373) and the gene has been cloned and sequenced (Argarana *et al* (1986) *Nucl. Acids Res.* 14, 1871-1872) and expressed in *E. coli* (Sano & Cantor (1990) *Proc. Natl. Acad. Sci. USA* 87, 142-146). A modified form of the gene is available commercially from British Bio-technology Ltd, Oxford, UK.

Of course, for the invention to work the cytotoxic portion may comprise intact streptavidin, or it may comprise a fragment or fragments of streptavidin retaining at least the biotin- and subunit-binding sites.

Of course, the cytotoxic portion may comprise other molecules which bind biotin with high affinity, such as intact avidin, or it may comprise a fragment or fragments of avidin retaining at least the biotin- and subunit-binding sites. A comparison of avidin and streptavidin is made in Table 3. As avidin is naturally glycosylated, then it may be desirable to express the DNA encoding the compound of the invention in a eukaryotic cell such as yeast, mammalian or insect cell.

	<u>Avidin</u>	<u>Streptavidin</u>
Source	Tissues and egg-whites of birds, reptiles and amphibia	<i>Streptomyces avidinii</i>
5 Glycoprotein	yes	no
pI	10	5
M _r (subunit)	67,000	60,000
Oligomeric state	Tetramer	Tetramer

- 10 By "subunit-binding sites" we mean those parts of the monomers that are necessary for the monomers to combine with one or more other monomers to produce an oligomer.

15 Biotin has an extremely high affinity for streptavidin ($K_d = 10^{-15}M$) and at the same time is small enough to diffuse rapidly through most tissues in the body. Some of the biotin conjugates useful in the invention are known in the art, and it is preferred that the biotin is conjugated via a flexible linker arm to reduce any steric hindrance to the binding of the biotin portion of the conjugate to streptavidin or avidin.

20 Examples of biotin conjugates useful in the invention are biotinylated growth factors and cytokines such as TNF α -biotin and EGF-biotin which are generally available from Boehringer Mannheim, Mannheim, Germany, and biotin-alkaline phosphatase, biotin-fluorescein, biotin-peroxidase and
 25 other conjugates generally available from Calbiochem-Novabiochem, Nottingham, UK. Activated biotin reagents, suitable for conjugating to other molecules, are generally available from Fluka, Buchs, Switzerland.

In a second preferred embodiment of the invention, the cytotoxic portion
 30 is a dimeric compound with ribonucleolytic activity, such as a ribozyme,

but preferably ribonuclease (RNase). The enzymes of the RNase family are able to degrade single-stranded RNA molecules to smaller polynucleotides and are directly cytotoxic when intracellular. Bovine seminal RNase (BSRNase) has activities in addition to its RNA-degrading activity, namely anti-tumour (Vescia *et al* (1980) *Cancer Res.* **40**, 3740-3744; Vescia & Tramontano (1981) *Mol. Cell. Biochem.* **36**, 125-128); immunosuppressive (Tamburrini *et al* (1990) *Eur. J. Biochem.* **190**, 145-148; activation by interferon- γ (Schein *et al* (1990) *Nucl. Acids Res.* **18**, 1057) and anti-spermatogenic (Doital & Matonsek (1973) *J. Reprod. Fertil.* **33**, 263-274). BSRNase is a dimer and forms two unique disulphide bridges across the subunit interface (Piccoli *et al* (1988) *Biochem J.* **253**, 329-336). The cDNA encoding the precursor to BSRNase can be prepared using the methods disclosed by Preub *et al* (1990) *FEBS Lett.* **270**, 229-232.

15

Of course, for the invention to work the cytotoxic portion may comprise intact BSRNase, or it may comprise a fragment or fragments of BSRNase retaining at least the active site and subunit-binding sites.

20 It is further preferred if the fusion with the RNase comprises the sequence KDEL (SEQ ID No 29) at, or near to, the C-terminus of the protein.

It is still further preferred if a linker sequence is present at the N-terminus of the RNase to allow the N-terminus to be more flexible and increase the
25 likelihood of dimer formation.

Preferably, a disulphide-loop-containing sequence which allows an RNase to be linked to a ScFv via a disulphide bond is present in a fusion protein.

30 In one embodiment the invention, the cytotoxic portion is a compound

with DNA endonucleolytic activity such as copper-phenanthroline adducts but preferably is a DNA endonuclease, for example deoxyribonuclease-I (DNase-I), which is an endonuclease which cleaves double-stranded DNA to yield 5' phosphorylated polynucleotides. It does not cut all DNA sites with the same frequency as it is affected by the local structure of the DNA (specifically, the size of the minor groove).

Alternatively, the DNA endonuclease could be a type II restriction endonuclease. Type II restriction endonucleases are enzymes isolated from microorganisms, usually bacteria, which cleave double-stranded DNA at specific sequences. Typically, the type II restriction endonucleases recognise palindromic sequences in DNA and cleave both strands of the DNA within or adjacent the recognition site. Type II restriction enzymes are dimers of identical subunits, and, for example, *EcoRI* is a homodimer of 31 kDa subunits which recognises the sequence 5'-GAATTC-3'.

Other type II restriction enzymes recognise different hexonucleotide sequences, for example *BamHI* recognises 5'-GGATCC-3', *HindIII* recognises 5'-AAGCTT-3'. In addition, type II restriction enzymes which recognise different numbers of bases are known, for example, *MspI* recognises 5'-CCGG-3', *Sau3AI* recognises 5'-GATC-3', *Hinfi* recognises 5'-GANTC-3' and *NotI* recognises 5'-GCGGCCGC-3'. Of course, the fewer specific bases in the recognition sequence, the more likely that any DNA molecule will be cleaved by the cognate type II endonuclease.

The gene for the bovine DNase I has been chemically synthesized and expressed in *E. coli* (Worrall & Connolly (1990) *J. Biol. Chem.* 265, 21889-21895. The gene for the human enzyme has been cloned, from a human pancreatic cDNA library constructed in λ gt10 and the enzyme has

been expressed in human cell culture and used in the relief of cystic fibrosis symptoms, by reducing the viscosity of sputum, by degrading the viscous DNA (Shak *et al* (1990) *Proc. Natl. Acad. Sci.* 87, 9188-9192; Hubbard *et al* (1992) *N. Engl. J. Med.* 326, 812-815). All the enzymes
5 are compact, monomeric proteins of about 29 kDa (260 amino acids); when glycosylated the human enzyme is about 35 kDa. It is dependent on divalent cations for activity (Ca^{2+} , Mg^{2+}). The human enzyme is about 75% identical to the bovine enzyme, at the amino acid sequence level. The synthetic gene encoding the bovine DNase-I can be prepared using the
10 methods disclosed by Worrall & Connolly (1990) *loc. cit.*

The enzyme from bovine pancreas has been purified and crystallized, and a high resolution structure determined at 2Å (Suck & Oefner (1986) *J. Mol. Biol.* 192, 605-632).

15 One aspect of the invention is the introduction into the targeted cell of the DNase I enzyme. During stages of mitosis, when the nuclear membrane is dissolved, the chromosomal DNA of the said targeted cell will be susceptible to nuclease attack. In this embodiment of the invention
20 DNase I will be particularly cytotoxic to rapidly dividing cells, such as tumour cells.

A further aspect of the invention is the incorporation into the compound of the invention a nuclear localisation sequence from the SV40 large T antigen (Kalderon *et al* (1984) *Cell* 39, 499-509). The said nuclear
25 localisation sequence is PKKKRKV (SEQ ID No 1), or analogues thereof, and a DNA fragment encoding the said sequence, or analogues thereof, may or may not be incorporated into the gene expressing the compound of the invention containing DNase I as the second part n.

Inclusion of the said nuclear localisation sequence will allow the compound of the invention to gain access to the chromosomal DNA during the periods of the cell cycle when the nuclear membrane is intact, as the nuclear pores are permeable to large macromolecules incorporating the
5 said nuclear localisation sequence, or analogues thereof.

For the invention to work, of course, the cytotoxic portion may comprise a fragment of RNase or of DNA endonuclease which retain their enzymatic activity, such as the active site, and in the case of the dimeric
10 RNase, and restriction endonuclease, their subunit binding site.

A further aspect of the invention is that the RNase and the DNase are of mammalian, preferably human, origin. The use of the said mammalian proteins as the second, functional portion of the compound of the
15 invention is advantageous as such compounds are less likely to give rise to undesirable immune reactions.

Many target cell-specific molecules are known, such as those disclosed in Table 1, which are not joined to a further directly or indirectly cytotoxic
20 portion, but may nevertheless be useful in directing cytotoxic agents to a target cell.

Thus in a further aspect of the invention a compound comprises a mediator portion and a directly or indirectly cytotoxic portion. The
25 mediator may recognise the native target cell-specific molecule, but it is preferable for the mediator to recognise a derivative of the said molecule.

In the case of antibodies, the native target cell-specific molecule may be recognised by the mediator via its Fc portion.

The said derivative may be made by joining a moiety, such as a small molecule, for example a hapten, to the said molecule, and may be recognised, if the mediator is, for example, an antibody or fragment thereof.

5

The advantage in using this method is that the same moiety may be joined to all types of target cell-specific molecules, and then only one compound, comprising a mediator which recognises the said moiety and a directly or indirectly cytotoxic portion, may be used to deliver the cytotoxic agent to
10 the target cell.

In one embodiment of the invention the mediator is ScFV_{NP}, and the moiety recognised by the said ScFV_{NP} is the hapten 4-hydroxy-3-nitrophenylacetic acid (NP) or 4-hydroxy-3-iodophenylacetic acid, and the
15 target cell-specific molecule is an antibody.

Other haptens are suitable as are other molecules, such as peptides, that can be recognised by the mediator. Conveniently the peptide is the core mucin peptide.

20

Before such molecules can be regarded as suitable candidates, there is a requirement that cell specificity be demonstrated and a further requirement that this specificity be shown to be conferred only by the combination of the interaction of the primary targeting antibody with target, and the
25 interaction of the second step reagent (in this case the ScFv) with the primary antibody. To this end, the primary antibody needs to be recognised specifically by the mediator, and therefore requires stable modifications that will distinguish it from native antibodies. Multiple derivatisation of the primary antibody with a hapten fulfils this demand,
30 and has the further advantage of amplification, providing an array of

secondary targets for the mediator.

Of course, other mediators such as Fab, F(ab')₂, dAbs or other antibody fragments may be used. The mediator may also recognise the moiety in
5 a non-immune sense, such as in biotin-streptavidin recognition. It is preferred if the moiety recognised is a small molecule, but the moiety may also be a polypeptide, peptide, oligosaccharide or the like.

The murine immune response to the haptens 4-hydroxy-3-nitrophenylacetic
10 acid (NP) and 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) is dominated by well characterised V_H domains and a λ_1 light chain (Kabat *et al* (1987) *Sequences of proteins of immunological interest*, US Department of Health and Human Services, Public Health Services, National Institutes of Health). NP-specific V_H domains have been used in
15 the construction of recombinant antibodies (Neuberger *et al* (1984) *Nature* 312, 604-608, Casadei *et al* (1990) *Proc. Natl. Acad. Sci. USA* 87, 2047-2051). The hapten itself is well studied and of some immunological interest (Brownstone *et al* (1966) *loc. cit.*) and is also available commercially in a variety of chemical forms. It is relatively simple to
20 conjugate NP or NIP to other proteins including antibodies.

We describe in the Examples the construction and characterisation of a ScFv with an affinity in the range of $1-3 \times 10^8 \text{ M}^{-1}$ at pH 7.4 for NIP conjugated to BSA, sufficiently high that the molecule is suitable as a
25 second step targeting reagent. Derivatisation with hapten resulted in reduction in immunoreactivity of the primary antibody, but even under these adverse circumstances the hapten-conjugated antibody was still capable of delivering ScFv_{NP} specifically to cells. Since about forty hapten molecules were conjugated, on average, to each mAb molecule, there is
30 still a potential 40-fold amplification provided. The specificity of targeting

is governed by the interactions of primary antibody with target, and the ScFv_{NP} with derivatised primary antibody, since the ScFv does not bind cells and non-derivatised antibodies bound at cells cannot capture the ScFv. The ScFv described here can therefore be considered as a universal
5 agent for delivery of drugs or radionuclides or other cytotoxic agents to any cell type for which a previously characterised antibody exists.

In this aspect of the invention, the cytotoxic portion joined to the mediator portion may be a drug, pro-drug, radionuclide, protein including an
10 enzyme, antibody or any other therapeutically useful reagent.

Thus, the drug may be a cytotoxic chemical compound such as methotrexate, adriamycin, vinca alkaloids (vincristine, vinblastine, etoposide), daunorubicin or other intercalating agents. The enzyme, or
15 enzymatic portion thereof, may be directly cytotoxic, such as DNaseI or RNase, or indirectly cytotoxic such as an enzyme which converts a substantially non-toxic pro-drug into a toxic form. The protein may be ricin. The cytotoxic portion may comprise a highly radioactive atom, such as iodine-131, rhenium-186, rhenium-188 or yttrium-90, which emits
20 enough energy to destroy neighbouring cells.

An indirectly cytotoxic portion may be a small-molecule binding site wherein the said small-molecule is capable of being conjugated to any from the following cytotoxic compounds: radioactive compound; drug;
25 pro-drug; radionuclide; protein including enzyme; antibody; or toxin.

We hereby disclose the principle that ScFvs are suitable for indirect targeting. Moderating the degree of derivatisation of the primary antibody will reduce the loss of immunoreactivity of the primary antibody whilst
30 still maintaining an array of secondary targets for the hapten-specific

ScFv.

In a further embodiment, the cytotoxic portion of the compound comprises at least the biotin-binding portion of streptavidin as disclosed in Example
5 4.

The compounds of the invention are administered in any suitable way, usually parenterally, for example intravenously, intraperitoneally or, preferably (for bladder cancer), intravesically (ie into the bladder), in
10 standard sterile, non-pyrogenic formulations of diluents and carriers, for example isotonic saline (when administered intravenously).

A further aspect of the invention provides a method of delivery of the compound of the invention which contains a binding site for a small
15 molecule, and the administration of the said small molecule conjugated with any from the following: drug, pro-drug, radionuclide, enzyme, antibody or any other therapeutically useful reagent, to give the "small molecule conjugate".

20 Once the compound has bound to the target cells and been cleared from the bloodstream (if necessary), which typically takes a day or so, the small molecule conjugate is administered, usually as a single infused dose. If needed, because the compound of the invention may be immunogenic, cyclosporin or some other immunosuppressant can be administered to
25 provide a longer period for treatment but usually this will not be necessary.

The timing between administrations of the compound and the small molecule conjugate may be optimised in a non-inventive way since target
30 cell/normal tissue ratios of conjugate (at least following intravenous

delivery) are highest after about 4-6 days, whereas at this time the absolute amount of antibody bound to the tumour, in terms of percent of injected dose per gram, is lower than at earlier times. Therefore, the optimum interval between administration of the conjugate and the small molecule conjugate will be a compromise between peak target concentration of enzyme and the best distribution ratio between target and normal tissues.

The dosage of the small molecule conjugate will be chosen by the physician according to the usual criteria. The dosage of the compound of the invention will similarly be chosen according to normal criteria, and, in the case of tumour treatment, particularly with reference to the type, stage and location of the tumour and the weight of the patient. The duration of treatment will depend in part upon the rapidity and extent of any immune reaction to the antibody or cytotoxic component of the compound.

A further aspect of the invention provides a method of delivery of the target cell-specific molecule and a compound of the invention which contains a mediator portion. Once the target cell-specific molecule has bound to the target cells and been cleared from the bloodstream (if necessary), which typically takes a day or so, the compound comprising a mediator portion is administered in any suitable way.

If the cytotoxic portion, joined to the mediator portion, contains a binding site for a small molecule, then, once the mediator-containing compound has bound to the target cell-specific molecule at the site of the target cell, and has been cleared from the bloodstream (if necessary), the said small molecule conjugate is administered as described *supra*.

The compounds of the invention either in themselves, or together with a target cell-specific molecule or additionally together with an appropriate toxic agent, capable of binding to the small molecule-binding site of the compound, are in principle suitable for the destruction of cells in any
5 tumour or other defined class of cells selectively exhibiting a recognisable (surface) entity. The compounds are principally intended for human use but could be used for treating other mammals including dogs, cats, cattle, horses, pigs and sheep.

10 The small molecule conjugate, when used in combination with a compound for diagnosis, usually comprises a radioactive atom for scintigraphic studies, for example technetium 99m (^{99m}Tc) or iodine-123 (^{123}I), or a spin label for nuclear magnetic resonance (nmr) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again,
15 iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

When used in combination with a compound for selective destruction of the tumour, the small molecule conjugate may comprise a highly
20 radioactive atom, such as iodine-131, rhenium-186, rhenium-188 or yttrium-90, which emits enough energy to destroy neighbouring cells, or a cytotoxic chemical compound such as methotrexate, adriamycin, vinca alkaloids (vincristine, vinblastine, etoposide), daunorubicin and other intercalating agents or (preferably) an enzyme or enzymatic portion thereof
25 which converts a non-toxic pro-drug into a toxic form. In the latter case, the compound of the invention is administered and, once there is an optimum balance between (i) the tumour to normal cell ratio of compound and (ii) the absolute level of compound associated with the tumour, the pro-drug is administered either systemically (eg intravenously) or
30 intravascularly, into the bladder. The enzyme/pro-drug systems of

Bagshawe and his co-workers may be used (*loc. cit.*) or the antibody-alkaline phosphatase conjugates, followed by etoposite phosphate (*loc. cit.*) or, more preferably, the cyanide-liberating systems described by Epenetos (*loc. cit.*).

5

The compounds of the invention, together with an appropriate small molecule conjugated to a readily-detectable reagent such as a radionuclide; fluorescent molecule; or enzyme are in principle suited for the recognition of antigens in other situations. These include immunoblotting procedures, such as the well-known Western blot (Towbin *et al* (1979) *Proc. Natl. Acad. Sci. USA* 76, 4350-4354); assays such as the enzyme-linked immunosorbent assay (ELISA); and *in situ* hybridisation experiments in which the presence of antigens within fixed cells is detected.

15 In a further embodiment of the invention, a compound comprising an oligomeric complex of at least two molecules each comprising a target cell-specific portion and a further portion wherein the molecules are complexed to one another via their further portions is useful in agglutinating cells. In a preferred embodiment the target cell-specific
20 portion of the compound of the invention recognises particular blood group antigens displayed on the surface of the erythrocyte, and because of the multivalent binding nature of the compound, the addition of the compound to blood may lead to haemagglutination. Thus, in this embodiment the compounds may be specific to particular antigens within
25 the ABO, Rhesus, Kell, or any other blood group systems, and the compound of the invention may find uses in blood group typing or other areas of tissue typing.

Antibodies, including monoclonal antibodies, are known which react with
30 most of the aforementioned blood group antigens, and it is well within the

scope of a person skilled in the art to derive, for example, ScFvs from such antibodies for use in the invention.

The invention will now be described in detail with reference to the
5 following figures and examples wherein:

Figure 1 shows a diagrammatic representation of compounds of the invention.

10 Figure 2 shows the construction of plasmids expressing ScFv_{NP}.

Figure 3 shows oligonucleotide primers used in the polymerase chain reaction to amplify various fragments of the ScFv coding region.

15 Figure 4 shows the nucleotide sequence (SEQ ID No 2) (and encoded protein sequence (SEQ ID No 3)) between the *Hind*III and *Eco*RI sites of pRAS107 and pRAS111.

Figure 5 shows the binding of a soluble protein expressed from pRAS111
20 to NIP₁₅-BSA.

Figure 6 shows that a soluble protein expressed from pRAS111 and which binds NIP₁₅-BSA can be competed by NIP₁₅-BSA.

25 Figure 7 shows the construction of plasmids expressing ScFv-streptavidin fusions *in vitro*.

Figure 8 shows the construction of plasmids for the expression of ScFv-streptavidin fusions in *E. coli*.

Figure 9 shows the nucleotide sequence (SEQ ID No 4) (and deduced amino acid sequence (SEQ ID No 5)) between the *Hind*III and *Eco*RI sites of pRAS108 and pRAS112.

- 5 Figure 10 shows the nucleotide sequence (SEQ ID No 6) (and deduced amino acid sequence (SEQ ID No 7)) between the *Hind*III and *Eco*RI sites of pRAS109 and pRAS113.

- 10 Figure 11 shows the nucleotide sequence (SEQ ID No 8) (and deduced amino acid sequence (SEQ ID No 9)) between the *Hind*III and *Eco*RI sites of pRAS110 and pRAS114.

Figure 12 shows the detection of soluble pRAS112-encoded protein (full length ScFv_{NP}-streptavidin monomer) in bacterial supernatants.

15

Figure 13 shows that pRAS112-encoded protein binds to NIP₁₅-BSA, but not to lysozyme.

- 20 Figure 14 shows that concentrated pRAS112-encoded protein binds iminobiotin-Sepharose at pH 11 in contrast to parental ScFv_{NP} protein that does not.

Figure 15 shows a diagrammatic representation of pRAS112-encoded protein.

25

Figure 16 shows the construction of plasmids expressing ScFv-BSRNase fusion molecules.

- 30 Figure 17 shows a diagrammatic representation of a ScFv-BSRNase heterodimer.

Figure 18 shows the construction of plasmids expressing ScFv-DNaseI fusion molecules.

Figure 19 shows the purification of pRAS111 ScFv_{NP} protein.

5

Figure 20 shows indirect targeting of pRAS111 ScFv_{NP}.

Figure 21 shows the nucleotide sequence (SEQ ID No 10) of the ScFv-BSRNase fusion (anti-4-OH-nitrophenacetyl antibody) that has been
10 inserted between the *Hind*III and *Eco*RI sites of plasmid pSP71.

Figure 22 shows the nucleotide sequence (SEQ ID No 11) of the ScFv-BSRNase fusion (H17-BSRNase; anti-human placental alkaline
15 phosphatase antibody; H17E2) that has been inserted between the *Hind*III and *Eco*RI sites of plasmid pSP71.

Figure 23 shows the nucleotide sequence (SEQ ID No 12) of the ScFv-BSRNase fusion (anti-lysozyme antibody) that has been inserted between
20 the *Hind*III and *Eco*RI sites of a plasmid pUC18.

Figure 24 shows the nucleotide sequence (SEQ ID No 13) of the ScFv-DNaseI fusion (anti-4-OH nitrophenacetyl antibody) that has been inserted
between the *Hind*III and *Bgl*II sites of plasmid pSP71.

25 Figure 25 shows the nucleotide sequence (SEQ ID No 14) of the ScFv-DNaseI fusion (anti-human placental alkaline phosphatase antibody; H17E2) that has been inserted between the *Hind*III and *Bgl*II sites of plasmid pSP71.

30 Figure 26 shows the nucleotide sequence (SEQ ID No 15) of the ScFv-

DNaseI fusion (anti-lysozyme antibody) that has been inserted between the *HindIII* and *BglII* sites of plasmid pUC18.

Figure 27 shows the results of cell-killing experiments using HEp2 cells and the fusion protein H17-DT-BSR, H17-DT-BSR/KDEL and H17-DT-BSR/KDELINK.

Figure 28 is a schematic diagram of the H17E2 scFv-seminal RNase fusion proteins. The plasmid which express them are named in parentheses.

Figure 29 shows the nucleotide sequence (SEQ ID No 24) encoding the H17E2 scFv-diphtheria toxin disulphide loop-BSRNase (H17-Dip. Tox.-BSRNase).

Figure 30 shows the nucleotide sequence (SEQ ID No 25) encoding the H17E2 scFv-diphtheria toxin disulphide loop-BSRNase-KDEL (H17-Dip. Tox.-BSRNase KDEL).

Figure 31 shows the nucleotide sequence (SEQ ID No 26) encoding the H17E2 ScFv diphtheria toxin disulphide loop-Linker-BSRNase-KDEL (H17-Dip. Tox.-link-BSRNase KDEL).

Figure 32 shows the nucleotide sequence (SEQ ID No 27) encoding the H17E2 ScFv-Linker-BSRNase-KDEL (H17-LBSRNase-KDEL).

Figure 33 shows the nucleotide sequence (SEQ ID No 28) encoding the H17E2 ScFv-BSRNase KDEL).

Figure 34 shows the elution of pRAS111 and pRAS112 proteins from NP-

sepharose with 50 mM glycine HCl, pH2.2.

Example 1: Construction of a single-chain Fv (ScFv) reactive against the hapten NP (4-OH nitrophenacetyl)

5

Plasmid constructions

- Plasmids are shown in Figure 2. Filled circles represent promoters: P_{lac} , *lac* promoter of pUC plasmids; P_{SP6} , SP6 promoter; P_{T7} , T7 promoter.
- 10 Open boxes represent fused gene portions: *pelB*, the signal sequence derived from the pectate lyase B gene of *Erwinia caratovora*; $(G_4S)_3$, flexible oligopeptide linker comprising three tandem repeats of N-GlyGlyGlyGlySer-C (SEQ ID No 16); myc, a small immunogenic tag derived from c-myc. Restriction enzyme sites: B, *Bam*HI; Bs, *Bst*EII; b, *Bgl*II; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xho*I.
- 15

- Plasmid pSWsFvD1.3myc (McCafferty *et al* (1990) *Nature* 348, 552-554) encodes a single-chain Fv reactive against hen egg lysozyme, and which
- 20 comprises $VH_{D1.3}$ and $V\kappa_{D1.3}$ domains linked by a flexible oligopeptide, $(G_4S)_3$, under the transcriptional control of the *lac* promoter of *E. coli*. The region encoding $V\kappa_{D1.3}$ was replaced by one encoding $V\lambda$ in the following manner. The segment encoding $VH_{D1.3}(G_4S)_3$ was subjected to polymerase chain reaction (PCR) mediated amplification using
- 25 oligonucleotide primers VHBACK2 (SEQ ID No 17) and BAMLINKERFOR (SEQ ID No 18) (Figure 3). Primer BAMLINKERFOR directs the incorporation of a *Bam*HI site that also encodes the two carboxy-terminal amino acids of the flexible oligopeptide linking the two V domains.

A V λ gene segment was amplified from chromosomal DNA of plasmacytoma J558L using primer pair BAMV λ BACK (SEQ ID No 19) and ECOV λ FOR (SEQ ID No 20). The former directs the incorporation of a *Bam*HI site at the 5' end of the gene; the latter two stop codons and
5 *Xho*I and *Eco*RI sites at the 3' end of the gene.

The two amplified products were used to replace the *Pst*I-*Eco*RI fragment of plasmid pRAS103 to generate plasmid pRAS106 which encodes a ScFv protein comprising VH_{D1.3}(G₄S)₃V λ _{J558L} under the transcriptional control
10 of the SP6 promoter.

The *Pst*I-*Bst*EII fragment of pRAS106 was replaced with a *Pst*I-*Bst*EII fragment encoding VH_{NP} amplified from plasmid pRAS49 (Spooner and Lord (1991) *loc. cit.*) using primers VHBAC3 (SEQ ID No 21) and
15 VH1FOR-2 (SEQ ID No 22) to generate plasmid pRAS107. This bears a VH_{NP}(G₄S)₃V λ _{J558L} ScFv under the transcriptional control of the SP6 promoter, and is intended purely for expression in *in vitro* systems.

Plasmid pRAS111 bears the ScFv of pRAS107, but under T7 promoter
20 control, and is suitable for expression in both *in vitro* systems and bacterial systems.

The nucleotide sequence (and deduced amino-acid sequence) between the *Hind*III and *Eco*RI sites of plasmids pRAS107 and pRAS111 are given in
25 Figure 4.

Table 3: Plasmids used

Plasmid	Relevant characteristics	Source or reference
pSWsFvD1.3myc	Anti-lysozyme ScFv, VH _{D1.3} (G ₄ S) ₃ Vk _{D1.3}	McCafferty <i>et al</i> (1990) <i>loc. cit.</i>
pRAS103	Anti-lysozyme ScFv-ricin A chain fusion, <i>lac</i> promoter	Spooner <i>et al</i> (1992) pp 7-15 in <i>Monoclonal Antibodies 2; Applications in Clinical Oncology</i> (Epenetos, A.A., Ed), Chapman & Hall
5 pRAS106	VH _{D1.3} (G ₄ S) ₃ Vλ _{J558L} , SP6 promoter	This application
pRAS49	Anti-NP antibody H chain-ricin A chain fusion, IgH promoter	Spooner and Lord (1991) <i>loc. cit.</i>
pRAS107	VH _{NP} (G ₄ S) ₃ Vλ _{J558L} , SP6 promoter	This application
pRAS111	VH _{NP} (G ₄ S) ₃ Vλ _{J558L} , T7 promoter	This application

10 Growth of plasmacytoma J558L and DNA preparation

Mouse plasmacytoma J558L cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Cells were washed twice in standard phosphate-buffered saline pH 7.4 (PBS) and high molecular weight DNA was prepared by addition, with gentle vortexing, of 10⁶ cells suspended in 100 μl PBS to 2.5 ml 10 mM Tris-HCl 1 mM EDTA pH 8.0 containing 0.02% (w/v) SDS. After adding Proteinase K to 1 mg.ml⁻¹, incubation (3h, 50°C) and two phenol/chloroform extractions, DNA was precipitated with ethanol, and dissolved overnight at 4°C in 1 ml 10 mM Tris-HCl 1 mM EDTA pH 8.

Polymerase chain reaction

Plasmid or chromosomal DNA (100 ng) was subjected to 24 rounds of PCR-mediated amplification (94°C, 1 min; 65°C, 1.5 min; and 72°C, 2 min) in 50 µl reaction volumes containing 25 pmol of each appropriate oligonucleotide primer, 250 µM of each dNTP, 67 mM Tris-HCl (pH 8.8), 17 mM (NH₄)₂SO₄, 1.5-6 mM MgCl₂, 200 mg.ml⁻¹ gelatin and 5 units of Taq polymerase (Cetus) overlaid with 25 µl paraffin oil. Amplified DNA was extracted once with phenol/chloroform and precipitated with ethanol before use.

Bacterial expression of pRAS111 protein

E. coli K12 JM109(DE3), a JM109 derivative with a chromosomal insertion of T7 polymerase under *lac* transcriptional control, was transformed with plasmid pRAS111. Cells were grown to a density of 10⁷ ml⁻¹ and expression of pRAS111 protein was induced by induction of T7 polymerase with 100 nM IPTG. A 31 kDa protein accumulates in the cells in sufficient quantity for provisional identification by Coomassie staining of cell extracts. The identity is confirmed by Western Blotting, probing with biotinylated goat anti-mouse lambda (Gαmλ) antiserum.

In addition, *E. coli* K12 BL21 (DE3), a derivative of BL21 with a single chromosomal copy of T7 RNA polymerase under *lacUV5* promoter control (Studier and Moffatt (1986) *J. Mol. Biol.* 189, 113-130) was transformed with plasmid pRAS111. Cultures (400 ml) were grown at 37°C or at room temperature in minimal salts medium supplemented with 100 µg.ml⁻¹ ampicillin and 1% glucose or in L-broth supplemented with 100 µg.ml⁻¹ ampicillin, to a density of 10⁷ cells.ml⁻¹. Expression of pRAS111 ScFv protein was achieved by induction of T7 polymerase with

100 nM IPTG. After induction, cells were grown for 24 h to permit accumulation of pRAS111 ScFv protein in the growth medium.

Biological activity and affinity purification of pRAS111 protein

5

Filtered bacterial supernatants were applied to wells of a 96-well plate previously coated with 10 mg.ml⁻¹NIP₁₅-BSA or 300 mg.ml⁻¹ hen egg lysozyme, and bound protein was detected by serial incubation with biotinylated G α m λ antiserum and HRPO-streptavidin conjugate. Colour changes were generated by incubation with ABTS and were monitored at 405 nm.

A soluble protein present in the growth medium of JM109 (DE3)/pRAS111 cultures, but not in cultures of JM109 (DE3), binds NIP₁₅-BSA, but not lysozyme (Figure 5). Filtered bacterial growth medium recovered after induction of pRAS111 protein was applied to wells of an ELISA plate coated with 10 μ g.ml⁻¹ NIP₁₅-BSA (⊙) or 300 μ g.ml⁻¹ hen egg lysozyme (◆). Bound protein was detected by serial incubation with biotinylated G α m λ (Goat anti-mouse lambda light chain) antisera and horseradish peroxidase conjugated streptavidin diluted in blocking buffer, and colour changes generated by addition of ABTS were monitored at 405 nm. A soluble protein present in the growth medium of JM109(DE3)/pRAS111 cultures, but not in cultures of JM109(DE3), binds NIP₁₅-BSA, can be competed with NIP₁₅-BSA (Figure 6). ScFv protein was allowed to bind ELISA wells coated with 10 μ g.ml⁻¹ NIP₁₅-BSA in the absence of competing hapten, or in the presence of 0.010 μ g.ml⁻¹ (◇), 0.019 μ g.ml⁻¹ (X), 0.039 μ g.ml⁻¹ (-), 0.078 μ g.ml⁻¹ (+), 0.156 μ g.ml⁻¹ (◆), 0.313 μ g.ml⁻¹ (*), 0.625 μ g.ml⁻¹ (Δ), 1.25 μ g.ml⁻¹ (■), 2.5 μ g.ml⁻¹ (□), 5 μ g.ml⁻¹ () or 10 μ g.ml⁻¹ (○) competing hapten. Bound protein

was detected by serial incubation with biotinylated G α m λ antisera and horseradish peroxidase conjugated streptavidin, and colour changes generated by addition of ABTS were monitored at 405 nm. The ScFv encoded by pRAS111 was found to have a binding affinity for NP of K_d
5 = 4×10^{-9} M. Since bivalency of an antibody commonly provides an extra three orders of magnitude of binding ability, then an avidity of at least 10^{12} M would be predicted for bivalent molecules derived from ScFvNP.

As an alternative, growth medium, filtered through 0.2 μ m nitrocellulose
10 filters to remove cells and particulates, was adjusted to 80% saturation with solid ammonium sulphate at 4°C. After incubation (4°C, 1 h) treated medium was centrifuged (10,000 x g, 30 min) to pellet insoluble proteins. Pellets were taken up in 20 ml PBS and were dialysed exhaustively against PBS at 4°C. Insoluble material after dialysis was
15 removed by brief centrifugation and the remainder was adjusted to 40 ml final volume with PBS, to 0.02% with sodium azide and was applied slowly (2 ml h⁻¹) to a 2 ml NP-Sepharose column at room temperature. After washing with 50 column volumes of PBS containing 0.02% sodium azide (PBS/azide), bound proteins were eluted with 50 mM glycine-HCl
20 pH 2.2 and fractions (2 ml) were immediately adjusted by addition of 200 μ l 2M unbuffered Tris base. Fractions containing ScFv protein were pooled, dialysed against PBS and concentrated using Macrosep (Amicon) concentrators with a 10 kDa cut-off. Yields were estimated by Bio-Rad protein assay, using rabbit IgG as a reference, and by absorbance at 280
25 nm assuming $A_{280}=1$ for 1.4 mg.ml⁻¹ solution.

Soluble NIP-binding activity was detected by ELISA analysis of bacterial growth medium after induction, and could be concentrated by ammonium sulphate precipitation and purified by affinity chromatography on NP-
30 Sepharose (Fig 19) so no attempt was made to recover pRAS111 ScFv

protein present in cell pellets. Yields of pRAS111 ScFv from growth medium were not greatly different when induced at room temperature or 37°C. Induction of expression was efficient in minimal salts medium and not discernible in rich broth; however, little difference was noted in the
5 final yields. The most important factor found here was the bacterial strain, with yields of ~1.3 mg l⁻¹ pRAS111 ScFv protein recovered from cultures of BL21(DE3)/pRAS111, approximately ten-fold greater than those obtained from cultures of JM109(DE3)/pRAS111.

10 Specificity of pRAS111 protein

The screening agent used here, biotinylated G α m λ antiserum, also detects the λ 1 light chain of anti-NP/NIP antibodies. It is therefore not possible to demonstrate specificity of pRAS111 ScFv protein for NP or NIP by
15 competition with anti-NP/NIP antibodies, but only by its ability to recognise NP/NIP. A soluble protein present in growth medium of JM109 (DE3)/pRAS111 cultures, but not in untransformed cultures of JM109 (DE3), binds NIP₁₆-BSA, but not lysozyme, and can be competed with NIP₁₆-BSA (Fig 5). This activity can be retained on NP-Sepharose
20 columns, from where it can be eluted. In addition, targeting studies demonstrate no cross-reaction with BSA, PEPY2-BSA, antibody or mammalian cells.

Affinity determinations

25

Results of affinity determinations using ELISA-based techniques are given in Table 4. Affinity of pRAS111 ScFv for NIP was estimated firstly by adapting the method of Mariani *et al* (1987) *Molec. Immunol.* 24, 297-303), determining the concentration of total added antibody giving half-
30 maximal binding (C_{50}) assuming $C_{50} = 1/K_{app}$, where K_{app} is the apparent

affinity constant. This approximation only holds true if the number of available binding sites per well is sufficiently low that their contribution is insignificant. Determinations of K_{app} should approach K_{actual} as the amount of antigen per well is reduced. Table 4 shows that a point is reached where similar values of K_{app} are derived ($K = 2-3 \times 10^8 M^{-1}$), representing the closest approximation that can be made using this method.

To confirm the accuracy of this approach, similar estimations of K were made using the method of Hogg *et al* (1987) *Molec. Immunol.* 24, 797-801) in the absence of competing antigen, by calculating the slope of the linear portion of a plot of $A_{450}/[ScFv_{NP}]$ v A_{450} , where $A_{450}/[ScFv_{NP}] = fKn - fK(A_{450})$, A_{450} is the absorbance at 450 nm, $[ScFv_{NP}]$ is the concentration of added $ScFv_{NP}$, n is the concentration of available binding sites and f is the valency of the $ScFv_{NP}$ for NIP. A value of 1 was assigned to f .

Table 4: Affinity determinations of pRAS111 $ScFv_{NP}$ protein

Antigen coat	concn (mg ml ⁻¹) of coating buffer	K (M ⁻¹) Mariani <i>et al</i> (1987), Hogg <i>et al</i> (1987)	
NIP ₁₆ BSA	5	2.5 (± 0.1) × 10 ⁹	1.6 (± 0.1) × 10 ⁹
NIP ₁₆ BSA	1	8.2 (± 1.5) × 10 ⁸	8.1 (± 0.7) × 10 ⁸
NIP ₄ BSA	10	2.9 (± 0.3) × 10 ⁸	1.2 (± 0.2) × 10 ⁸
NIP ₄ BSA	5	2.5 (± 0.5) × 10 ⁸	1.8 (± 0.1) × 10 ⁸

Preparation of NP-Sepharose

Sephacrose support (20 ml) with an amine function (Affigel 102, Biorad) was washed and suspended by addition of 20 ml 40 mM triethylamine.

To this was added 430 mg NP-cap-OSu (Cambridge Research Biochemicals) dissolved in 1 ml dimethylformamide (DMF). After mixing by gentle inversion (2h, room temperature) and extensive washing in water and then PBS, NP-Sepharose was equilibrated in PBS/azide and stored in the dark at 4°C.

Western blots to identify pRAS111 protein

Western blots were performed as previously described (Spooner and Lord (1991) pp 65-77 in *Monoclonal Antibodies; Applications in Clinical Oncology* (Epenetos, A.A., Ed) Chapman and Hall) and pRAS111 ScFv protein was identified by serial incubations in PBS/5% milk powder/0.1% Tween 20 (blocking solution), biotinylated G α m λ antisera and streptavidin-HRPO diluted in blocking solution to concentrations recommended by the suppliers. After each incubation, blots were washed 5 times in PBS/0.1% Tween 20. Proteins bound by biotinylated G α m λ antisera and streptavidin-HRPO were revealed by incubation with DAB.

Example 2: Derivatisation of proteins with hapten

NIP-cap-OSu (Cambridge Research Biochemicals) was dissolved in dimethylformamide to 20 mg.ml⁻¹ and added to proteins as below.

NIP-BSA: for low coupling ratio, 80 μ l 20 mg.ml⁻¹ NIP-cap-OSu/DMF was added to 1 ml 200 mg.ml⁻¹ BSA in 10 mM triethylamine. For high coupling ratio, 800 μ l 20 mg.ml⁻¹ NIP-cap-OSu/DMF was added to 1 ml 200 mg.ml⁻¹ BSA in 100 mM triethylamine.

NIP-antibody: 200 μ l 20 mg.ml⁻¹ NIP-cap-OSu/DMF was added to 2 ml 2.8 mg.ml⁻¹ antibody (AUA1 or HMFG1, Unipath) in PBS/40 mM

triethylamine.

After mixing by inversion (2 h, room temperature) and extensive dialysis against PBS, insoluble material was removed by centrifugation. Soluble NIP-BSA was adjusted to 0.02% with sodium azide. Soluble NIP-antibody was sterilised by filtration (0.2 μ m filter). Haptenated proteins were stored at 4°C in the dark. Protein concentration was estimated by Bio-Rad protein assay.

- 10 The number of haptens conjugated to each protein molecule was estimated by absorbance at 430 nm according to Brownstone *et al* (1966) *Immunology* 10, 465-479: low coupling ratio NIP-BSA, 3.7 (NIP₄-BSA); high coupling ratio NIP-BSA, 16.4 (NIP₁₆-BSA); NIP-AUA1, 38.3 (NIP₃₈-AUA1) and NIP-HMFG1, 35.4 (NIP₃₅-HMFG1).

15

Example 3: Indirect targeting using pRAS111 ScFv protein

- The measured affinity of ScFv_{NP} or pRAS111 protein is sufficiently high to contemplate cell targeting by a two-step approach. Cells (LOVO and HT29) and peptide (PEPY-BSA) were incubated with AUA1, NIP₃₈-AUA1, or NIP₃₅-HMFG1, and bound material was detected by incubation with sheep anti mouse antisera (Sh α m) conjugated to HRPO or by serial incubation with biotinylated G α m λ and streptavidin-HRPO (Fig 20). LOVO cells, which express AUA1 antigen, can be identified by serial incubation with specific antibody (AUA1) and with Sh α m conjugated to HRPO. Hapten-derivatised NIP₃₈-AUA1 displayed a marked reduction in cell-binding ability, with loss of approximately 90% of immunoreactivity. Hapten-conjugated NIP₃₅-HMFG1 also bound LOVO cells, reflecting the ability of HMFG1 to bind these cells when presented at high concentration. When pRAS111 ScFv_{NP} protein was used as a detection
- 20
- 25
- 30

layer, hapten-derivatised NIP₃₈-AUA1 and NIP₃₅-HMFG1 were both recognised, but non-hapten-conjugated AUA1 was not. Similar results were obtained with a different cell line, HT29, that also expresses AUA1 antigen.

5

When the specificity of the system was altered completely, a peptide (PEPY2) derived from the protein backbone of polymorphic epithelial mucin identified with NIP₃₅-HMFG1 antibody was bound by pRAS111 ScFv protein whilst those incubated with AUA1 and NIP₃₈-AUA1 were not.

10

The specificity of pRAS111 ScFv_{NP} protein is therefore dependent upon prior targeting with a hapten-derivatised primary targeting vehicle, and the specificity of targeting depends only upon the interaction of primary hapten-conjugated targeting vehicle and the interaction of second step ScFv with the primary targeting vehicle.

15

For ELISAs using fixed mammalian cells, cells were seeded into wells of 96-well microculture plates at 10^5 cells.ml⁻¹ in RPMI supplemented with 10% foetal calf serum and were grown to confluence at 37°C in a 5% CO₂ atmosphere. Cells were washed twice in PBS, were incubated in 0.25% glutaraldehyde in PBS (100 µl per well, room temperature, 15 min) and after a further wash in PBS, were stored at 4°C in PBS/azide.

20

Unbound sites were blocked (30 min, room temperature) using 1% milk powder reconstituted in PBS containing 0.1% Tween 20 (blocking buffer). Antibodies and hapten-conjugated antibodies were applied and were detected by serial incubation with pRAS111 ScFv protein, biotinylated Gαmλ antisera and streptavidin-HRPO or by incubation with horseradish peroxidase conjugated Sheep anti mouse serum, diluted in blocking buffer

30

to appropriate concentrations. After each incubation, plates were washed 5 times in PBS containing 0.1% Tween 20. Colour changes were generated using ABTS (monitored at 405 nm) or OPD (monitored at 450 nm).

5

The results of indirect targeting of pRAS111 ScFv_{NP} are shown in Figure 20.

Binding of AUA1 (open circles), NIP₃₈-AUA1 (closed circles) and NIP₃₅-HMFG1 (open triangles) to LOVO cells, HT29 cells and to a peptide derived from the mucin backbone conjugated to BSA (PEPY2-BSA). Bound primary antibody was detected using HRPO-conjugated sheep anti-mouse antisera (Shoam) or by recognition using pRAS111 ScFv_{NP} (ScFv).

15 Example 4: Construction of high avidity ScFv-streptavidin fusion

Plasmid constructions

Plasmids for the *in vitro* expression of ScFv-streptavidin fusions are shown in Figure 7. Filled circles represent promoters: P_{SP6}, SP6 promoter; P_{T7}, T7 promoter. Open boxes represent fused gene portions: *pelB*, the signal sequence derived from the pectate lyase B gene of *Erwinia caratovora*; (G₄S)₃, flexible oligopeptide linker comprising three tandem repeats of N-GlyGlyGlyGlySer-C; A-P, a novel flexible oligopeptide linker.

25

Restriction enzyme sites: B, *Bam*HI; Bs, *Bst*EII; b, *Bgl*II; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xho*I; Xb, *Xba*I.

30 Plasmids for the expression of ScFv-streptavidin fusions in *E. coli* are

shown in Figure 8. Filled circles represent promoters: P_{SP6} , SP6 promoter; P_{T7} , T7 promoter. Open boxes represent fused gene portions: *pelB*, the signal sequence derived from the pectate lyase B gene of *Erwinia caratovora*; $(G_4S)_3$, flexible oligopeptide linker comprising three tandem repeats of N-GlyGlyGlyGlySer-C; A-P, a novel flexible oligopeptide linker Restriction enzyme sites: B, *Bam*HI; Bs, *Bst*EII; b, *Bgl*II; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xho*I; Xb, *Xba*I.

10 Segments of DNA encoding mature streptavidin monomers or fragments were amplified by PCR and were used to replace the *Xho*I-*Eco*RI fragment of plasmid pRAS107 to generate plasmids pRAS108, pRAS109 and pRAS110, which encode ScFv_{NP}-streptavidin fusions under SP6 transcriptional control.

15 Plasmid pRAS108 encodes a ScFv_{NP} fused via a novel oligopeptide (APAAAPA (SEQ ID No 23)). Its product is expected to tetramerise via the streptavidin monomer moieties. Mature streptavidin often forms higher order complexes, probably through interaction of the amino-terminal and carboxy-terminal regions which are thought to be flexible extensions. Many commercial preparations lack these, through natural proteolysis, and form stable tetramers. In order to mimic this, two further ScFv_{NP}-streptavidin derivatives were made, one borne on plasmid pRAS109 and which lacks the 19 carboxy terminal amino acids of streptavidin, and the other, on plasmid pRAS110, which further lacks the 25 12 amino-terminal amino acids of streptavidin. Plasmid pRAS110 thus encodes a ScFv_{NP} linked to "core" streptavidin monomers, typical of many commercial preparations.

30 Plasmids pRAS112, pRAS113 and pRAS114 are derived from plasmids

pRAS108, pRAS109 and pRAS110 respectively, and code for ScFv_{NP}-streptavidin fusions under the transcriptional control of the T7 promoter.

5 The nucleotide sequence (and deduced amino-acid sequence) between the *Hind*III and *Eco*RI sites of plasmids pRAS108 and pRAS112 are given in Figure 9, the sequences of plasmids pRAS109 and pRAS113 in Figure 10 and those of plasmid pRAS110 and pRAS114 are displayed in Figure 11.

10 Bacterial expression of pRAS112, pRAS113 and pRAS114 proteins

In contrast to ScFv_{NP}, in the conditions used, proteins encoded by plasmids pRAS112, pRAS113 and pRAS114 do not accumulate after induction in amounts sufficient for provisional identification by Coomassie staining. Western Blotting of cell extracts and culture supernatants, probing with 15 biotinylated G α m λ antiserum and HRPO-streptavidin conjugate or rabbit α -streptavidin (R α S) antiserum and HRPO-donkey α -rabbit (D α R) antiserum allows identification of fusion proteins of expected monomeric sizes. Very little ScFv_{NP}-core streptavidin accumulates after induction of expression of pRAS114 protein.

20 In non-reducing conditions, almost all of the ScFv-streptavidin material migrates with sizes corresponding to multimeric forms (at ~90 kDa for a dimer and 180 kDa for the tetramer). Note that in the conditions employed here, streptavidin itself exists mostly as higher order aggregates.

25

Antigen binding

30 Filtered bacterial supernatants were applied to wells of a 96-well plate previously coated with 10 μ g.ml⁻¹ NIP₁₅-BSA or 300 μ g.ml⁻¹ hen egg lysozyme, and bound protein was detected by serial incubation with

biotinylated $\text{G}\alpha\text{m}\lambda$ antiserum and HRPO-streptavidin conjugate or R α S antiserum and HRPO-D α R antiserum. Colour changes were generated by incubation with ABTS and were monitored at 405 nm.

- 5 Only soluble pRAS112 protein (full length ScFv_{NP}-streptavidin monomer) can be detected in bacterial supernatants (Figure 12). Filtered bacterial growth medium recovered after induction of pRAS112 (⊙), pRAS113 (◆) or pRAS114 (■) protein was diluted in PBS and applied to wells of an ELISA plate coated with 10 $\mu\text{g}.\text{ml}^{-1}$ NIP₁₅-BSA. Bound protein was
10 detected by serial incubation with Rabbit α Streptavidin antisera and horseradish peroxidase conjugated Donkey α Rabbit antisera, and colour changes generated by addition of ABTS were monitored at 405 nm. Like the parental ScFv_{NP}, this protein binds NIP₁₅-BSA, but not lysozyme (Figure 13). Filtered bacterial growth medium recovered after induction
15 of pRAS112 protein was applied to wells of an ELISA plate coated with 10 $\mu\text{g}.\text{ml}^{-1}$ NIP₁₅-BSA (⊙) or 300 $\mu\text{g}.\text{ml}^{-1}$ hen egg lysozyme (◆). Bound protein was detected by serial incubation with Rabbit α Streptavidin antisera and horseradish peroxidase conjugated Donkey α Rabbit antisera, and colour changes generated by addition of ABTS were monitored at 405
20 nm.

Partial purification of pRAS112 protein

- ScFv_{NP}-streptavidin fusion protein (pRAS112 protein) can be concentrated
25 about 20-fold by precipitation from 50% saturated ammonium sulphate and dialysis against PBS. As expected concentrated pRAS112 protein binds iminobiotin-Sepharose at pH11, in contrast to parental ScFv_{NP} protein (Figure 14). Concentrated proteins resolubilised in PBS after precipitation from 50% (pRAS112) or 80% (pRAS111) saturated ammonium sulphate
30 were applied at pH11 to a iminobiotin-Sepharose column (Pierce), and

antigen binding ability of material applied to the column (⊙) and material flowing through the column (♦) were measured by appropriate ELISA.
a) pRAS112 protein, b) pRAS111 protein.

5 The avidity of streptavidin fusions can be compared with univalent ScFvs.

a) The slope of a NIP-specific ELISA performed using pRAS112 streptavidin fusion differs from that performed using pRAS111 scFv.

10

b) pRAS112 protein binding to NIP-BSA cannot be competed with free NP, free NIP or NIP-BSA, whereas pRAS111 scFv can.

15

c) pRAS112 protein cannot be eluted in a single pulse from a NP-Sepharose column. Multiple pulses of low pH interspersed with high pH washes are required to elute this protein. In contrast, pRAS111 scFv elutes with a single low pH step (Figure 34).

20 This indicates that the streptavidin fusions (pRAS112) are binding multivalently.

A representation of the pRAS112 protein is shown in Figure 15.

25 **Example 5: Construction of ScFv-BSRNase fusion molecules**

Plasmid construction

Plasmids for the expression of ScFv-BSRNase fusions are shown in Figure 16. The plasmid pRAS111 is described in Example 1, and the plasmid pBSV5 is as described in Schein *et al*, *loc. cit*.

30

Figure 21 shows the sequence of the ScFv-BSRNase fusion (4-OH nitrophenacetyl antibody) inserted between the *Hind*III and *Eco*RI sites of plasmid pSP7 (available from Promega) to give plasmid pSPNPBSR as shown in Figure 16.

5

Figure 22 shows the sequence of the ScFv-BSRNase fusion (anti-human placental alkaline phosphatase antibody; H17E2) inserted between the *Hind*III and *Eco*RI sites of plasmid pSP71 to give plasmid pSPH17ΔXBSR as shown in Figure 16.

10

The amino acid sequences of the V_H and V_L chains of H17E2 are disclosed in "Monoclonal Antibodies - applications in clinical oncology", pages 37-43, 1991, A.A. Epenetos, ed., Chapman & Hall, UK.

15 Figure 23 shows the sequence of the ScFv-BSRNase fusion (anti-lysozyme antibody) inserted between the *Hind*III and *Eco*RI sites of plasmid pUC18 (available from Pharmacia) to give pUCD1.3BSR as shown in Figure 16.

Figure 17 shows a diagrammatic representation of the specific case where
20 a heterodimer has been synthesised and purified (as described *supra*), in this case each of the ScFvs recognises a different antigen on the same tumour cell.

The plasmids were made using standard methods of molecular biology as
25 disclosed by Sambrook *et al* (1989) in *Molecular Cloning, a laboratory manual*, 2nd Edn, Cold Spring Harbor Laboratory Press, NY, USA.

The plasmid pSPNPBSR encodes a protein which directs cytotoxin RNase to a target cell-specific molecule derivatised with NP or NIP. The
30 plasmid pPSH17ΔXBSR encodes a protein which directs RNase to cells

expressing the human placental alkaline phosphatase antigen. The ScFv encoded by this plasmid is derived from the monoclonal antibody H17E2 (see Table 1).

5 In addition to the fusion gene consisting of the H17E2 scFv and seminal RNase only (see above) the following fusion genes which incorporate one or more of the following are useful:

- 10 (i) A C-terminal "KDEL" sequence (endoplasmic reticulum retention signal), which may elevate cytotoxicity by increasing the retention of the protein in the cell and reducing its loss to other endosomal pathways.
- (ii) A linker sequence at the N-terminus of the RNase to allow the N-terminus to be more flexible and increase the likelihood of forming dimers.
- 15 (iii) A disulphide loop containing sequence, derived from the diphtheria toxin, which allows the scFv and RNase to be linked via a disulphide bond, and permits efficient release of the RNase from the scFv once the cytotoxin has been internalised.

20

The plasmids which contain these genes (described diagrammatically in Figure 28 and individual nucleotide sequences encoding these proteins given in Figures 29 to 33) are identical to that expressing the original scFv-RNase fusion protein, ie only the DNA sequence of the actual
25 cytotoxic molecule has been altered. The conditions for expression and refolding are as described in the earlier Examples.

Characterisation of the scFv-RNase protein

RNase activity of the fusion proteins.

All the fusions described, H17-BSRNase, H17-DT-BSRNase, H17-DT-BSRNaseKDEL, H17-DT-Link-BSRNase, H17-DT-Link-BSRNaseKDEL, H17-BSRNaseKDEL, H17-Link-BSRNaseKDEL, have RNA-degrading activity, as demonstrated by an RNase assay which involves incubating a sample of the refolded protein (10-50 ng of crude fusion protein) with 5 μ g of RNA in a volume of 20 μ l at 37°C for 1 hr. In each case all the RNA was degraded, showing qualitative RNase activity in the fusion protein preparations.

Antigen-binding activity of fusion proteins.

All the fusion proteins demonstrate binding to the antigen human placental alkaline phosphatase (hPLAP) in an ELISA system. The detecting layers for the ELISA were anti-bovine seminal RNase polyclonal antibodies (from rabbit) and anti-rabbit polyclonal antibodies (from goat).

Evidence for the dimeric nature of the scFv-RNase.

Gel filtration experiments show the native molecular weight of the fusion proteins. Data from binding experiments indicates that the molecule has a higher avidity than the single-chain H17E2 antibody alone: the scFv will bind to an antigen affinity column (the antigen is placental alkaline phosphatase) and is eluted with a buffer consisting of 50 mM diethylamine (DEA), pH 12. The fusion protein, due to its higher avidity cannot be eluted under these mild conditions, and more harsh conditions are needed, eg 100 mM glycine, pH 2.2. Also, when the scFv and whole IgG H17E2 and fusion proteins are bound to their antigen on an ELISA plate and washed with copious amounts of 50 mM DEA, over 90% of the scFv is washed off, whereas only 40% of the whole IgG and fusion protein is

washed off. Finally, the shape of the ELISA curve for the whole IgG H17EE2 and fusion protein are similar (shallow slope), but that of the scFv is a steep slope. These experiments indicate that the scFv-RNase protein is dimeric.

5

Cytotoxicity of the fusion proteins towards an antigen-positive cell-line (HEp2).

HEp2 cells were seeded in 96-well microtitre plates and grown overnight in E4 culture media with 10 foetal calf serum (FCS) at a density of 10^5 cells per well. The next day, 10 μ l of crude refolded fusion protein in PBS was added to each well and allowed to grow for 72 hr. Cell-killing was detected using the Promega cell-titre 96 assay kit, which measures cell proliferation.

15 The scFv-BSRNase fusion protein consisting of a disulphide loop, KDEL and linker showed significant cell killing activity. The estimated final concentration of the cytotoxin was between 10-100 nM (see Figure 27 for the results of these experiments).

20 **Example 6: Construction of ScFv-DNaseI fusion molecules without a nuclear localization signal**

Plasmids for the expression of ScFv-DNaseI fusions are shown in Figure 18. The plasmid pRAS111 is described in Example 1 and
25 M13mp19DNaseRec5 is described in Worrall and Connolly, *loc. cit.*

Figure 24 shows the sequence of the ScFv-DNaseI fusion (4-OH nitrophenacetyl antibody) inserted between the *Hind*III and *Bgl*II sites of plasmid pSP71 to give plasmid pSPNPDN1 as shown in Figure 18.

30

Figure 25 shows the sequence of the ScFv-DNaseI fusion (anti-human placental alkaline phosphatase antibody; H17E2) inserted between the *HindIII* and *BglII* sites of plasmid pSP71 to give plasmid pSPH17 Δ XDN1 as shown in Figure 18.

5

Figure 26 shows the sequence of the ScFv-DNase fusion (anti-lysozyme antibody) inserted between the *HindIII* and *BglII* sites of plasmid pUC18 to give pUCD1.3DN1 as shown in Figure 18.

- 10 The plasmids were made using standard methods of molecular biology as disclosed in Sambrook *et al* (1989) in *Molecular Cloning, a laboratory manual*, 2nd Edn, Cold Spring Harbor Laboratory Press, NY, USA.

15 The plasmid pSPNPDN1 encodes a protein which directs DNaseI to a target cell-specific molecule derivatised with NP or NIP.

The plasmid pSPH17 Δ XDN1 encodes a protein which directs DNaseI to cells expressing the human placental alkaline phosphatase antigen. The ScFv encoded by this plasmid is derived from the monoclonal antibody
20 H17E2 (see Table 1).

The scFv-DNase I fusion has been expressed under identical conditions to that of the RNase fusions and refolded. The crude refolded preparation of the scFv-DNase I fusion protein shows PLAP-antigen binding activity
25 in an ELISA system similar to the parent antibody H17E2. The detecting layers are anti-bovine DNase I (from rabbit) and anti-rabbit (from goat). The DNase I fusion protein also demonstrates DNA-degrading activity, in a similar system as that of the RNase assay, except 2 μ g of DNA is incubated. The activity is only present when 10 mM CaCl₂ and 4 mM
30 MgCl₂ is added, as is found with the naturally occurring bovine DNase I.

suggesting that functional scFv-DNase fusion molecules have been expressed and refolded from *E. coli*.

CLAIMS

1. A compound comprising a target cell-specific portion and a cytotoxic portion characterised in that the cytotoxic portion has nucleolytic activity.
5
2. A compound according to Claim 1 wherein the cytotoxic portion has ribonucleolytic activity.
- 10 3. A compound according to Claim 2 wherein the cytotoxic portion is a ribonuclease.
4. A compound according to Claim 3 wherein the ribonuclease is dimeric.
15
5. A compound according to Claim 4 wherein the ribonuclease is mammalian seminal ribonuclease.
6. A compound according to Claim 1 wherein the cytotoxic portion has DNA endonucleolytic activity.
20
7. A compound according to Claim 6 wherein the cytotoxic portion is at least the catalytically active portion of a DNA endonuclease.
- 25 8. A compound according to Claim 7 wherein the endonuclease is a mammalian deoxyribonuclease I.
9. A compound according to Claim 8 wherein a nuclear localization signal is incorporated.
30

10. A compound according to Claim 9 wherein the nuclear localization signal comprises the sequence PKKKRKV.
- 5 11. A compound according to Claim 7 wherein the DNA endonuclease is a restriction endonuclease.
- 10 12. A compound comprising a target cell-specific portion and a directly or indirectly cytotoxic second portion, characterised in that the target cell-specific portion recognises the target cell with high avidity.
- 15 13. A compound comprising a target cell-specific portion and a directly or indirectly cytotoxic portion characterised in that the cytotoxic portion is a sub-unit of an oligomer provided that, if the sub-unit is complexed with another sub-unit of the said oligomer then the said other sub-unit is the cytotoxic portion of a second compound of the invention.
- 20 14. A compound comprising an oligomeric complex of at least two molecules each comprising a target cell-specific portion and a further portion wherein the molecules are complexed to one another via their further portions.
- 25 15. A compound according to Claim 14 wherein the target cell-specific portion recognises a blood group antigen.
- 30 16. A compound comprising an oligomeric complex of at least two molecules each comprising a target cell-specific portion wherein the molecules are complexed to one another via their cytotoxic portions.

17. A compound according to Claim 16 wherein the respective cytotoxic portions of at least two of the molecules of the oligomeric complex are different from one another.
- 5 18. A compound according to Claim 16 wherein the respective target cell-specific portions of at least two of the molecules of the oligomeric complex are different from one another.
- 10 19. A compound comprising a target cell-specific portion and a directly or indirectly cytotoxic portion characterised in that the cytotoxic portion contains a binding site for a small molecule, wherein the said small-molecule binding site binds but does not modify catalytically the said small molecule.
- 15 20. A compound according to Claim 19 wherein the small-molecule binding site recognises the said small molecule with an affinity of at least $K_D = 10^{-12}M$.
- 20 21. A compound comprising a target cell-specific portion and a directly or indirectly cytotoxic portion characterised in that the target cell-specific portion comprises two or more binding sites for the target cell, wherein the target cell-specific portion is not an antibody or bivalent fragment thereof having respective arms which recognise the same entity as one another.
- 25 22. A compound according to any one of the preceding claims wherein the target cell-specific portion comprises an antibody or part thereof.
- 30 23. A compound according to any one of the preceding claims wherein the target cell-specific portion comprises a ScFv antibody fragment.

24. A compound according to any one of the preceding claims wherein the target cell-specific portion recognises and selectively binds to a tumour cell antigen.
- 5 25. A compound comprising a mediator portion and a directly or indirectly cytotoxic portion wherein the mediator portion recognises a target cell-specific molecule.
- 10 26. A compound according to Claim 25 wherein the target cell-specific molecule comprises a hapten and the mediator portion recognises the said hapten.
27. A compound according to Claim 26 wherein the said hapten is capable of chemical conjugation to the target cell-specific molecules.
- 15 28. A compound according to Claim 27 wherein the hapten is 4-hydroxy-3-nitrophenylacetic acid or 4-hydroxy-3-iodophenylacetic acid.
- 20 29. A compound according to any one of Claims 26 to 28 wherein the mediator is a ScFv.
30. A compound according to any one of Claims 26 to 29 wherein the cytotoxic portion comprises at least the biotin-binding portion of streptavidin.
- 25 31. A compound according to any one of Claims 26 to 29 wherein the cytotoxic portion has DNA endonucleolytic activity.
- 30 32. A compound according to Claim 31 wherein the cytotoxic portion

is at least the catalytically active portion of a DNA endonuclease.

- 5 33. A compound according to Claim 19 wherein the cytotoxic portion comprises at least the biotin- and subunit-binding portions of streptavidin.
- 10 34. A compound according to any of the preceding claims wherein the target cell-specific and cytotoxic portions of the compound are polypeptides.
- 15 35. A compound according to any of the preceding claims wherein the target cell-specific and cytotoxic portions of the compound are fused.
- 20 36. A nucleotide sequence encoding a compound according to Claim 35.
- 25 37. A therapeutic system comprising a compound according to Claim 19 and a drug, pro-drug, radionuclide, enzyme, antibody, toxin or any other reagent which is conjugated to the said small molecule.
- 30 38. A therapeutic system according to Claim 37 wherein the said small molecule is biotin.
- 35 39. A therapeutic system comprising a compound according to Claim 25 and a target cell-specific molecule.
- 40 40. A therapeutic system comprising a compound according to Claim 30, a target cell-specific molecule and a drug, pro-drug, radionuclide, enzyme, antibody toxin or any other reagent which is conjugated to biotin.

41. A pharmaceutical composition comprising a compound according to any one of Claims 1 to 35 and a pharmaceutical carrier.
- 5 42. A method of treating a mammal having target cells to be destroyed, the method comprising (1) administering a compound according to any one of Claims 12, 13 and 16 to 21, wherein the cytotoxic portion is indirectly cytotoxic to the mammal, (2) allowing the ratio of (compound bound to the target cells) : (compound not bound to the target cells) to reach a desired value, and (3) administering a
10 drug, pro-drug, radionuclide, enzyme, antibody, toxin or any other reagent which is conjugated to a small molecule.
43. A method of treating a mammal according to Claim 42 wherein the said small molecule is biotin.
15
44. A method of treating a mammal having target cells to be destroyed, the method comprising administering the compound according to any one of Claims 1 to 13 and 16 to 24 wherein the cytotoxic portion is directly cytotoxic.
20
45. A method of treating a mammal having target cells to be destroyed, the method comprising administering a target cell-specific molecule, (2) allowing the ratio of (molecule bound to the target cells):(molecule not bound to the target cells) to reach a desired
25 value, (3) administering a compound according to Claim 30 wherein the cytotoxic portion is indirectly cytotoxic, (4) allowing the ratio of (compound bound to target cell-specific molecule):(compound not bound to target cell-specific molecule) to reach a desired value and (5) administering a drug, pro-drug, radionuclide, enzyme, antibody,
30 toxin or any other reagent which is conjugated to biotin.

46. A method of treating a mammal having target cells to be destroyed, the method comprising administering a target cell-specific molecule, (2) allowing the ratio of (molecule bound to target cells):(molecule not bound to target cells) to reach a desired value, and (3) administering a compound according to any one of Claims 25 to 29 wherein the cytotoxic portion is directly cytotoxic.
47. A method of determining a blood group of an individual, the method comprising (1) removing blood from an individual into a container, (2) adding a compound according to Claim 15 and (3) observing the extent of haemagglutination.

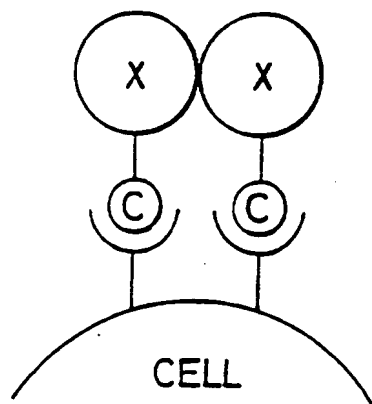


Fig. 1(a)

Fig. 1(b)

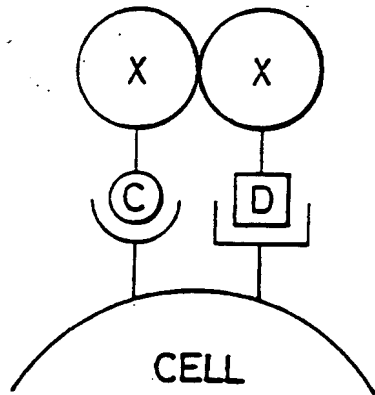
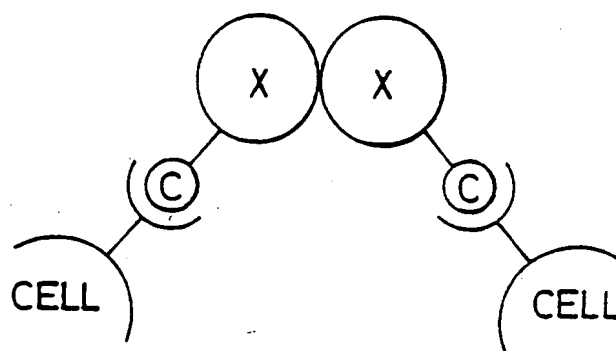
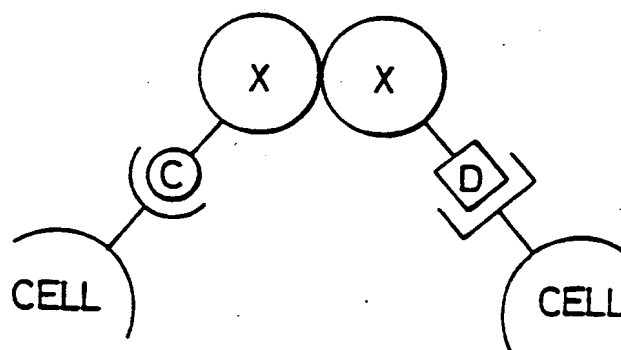


Fig. 1(c)

Fig. 1(d)



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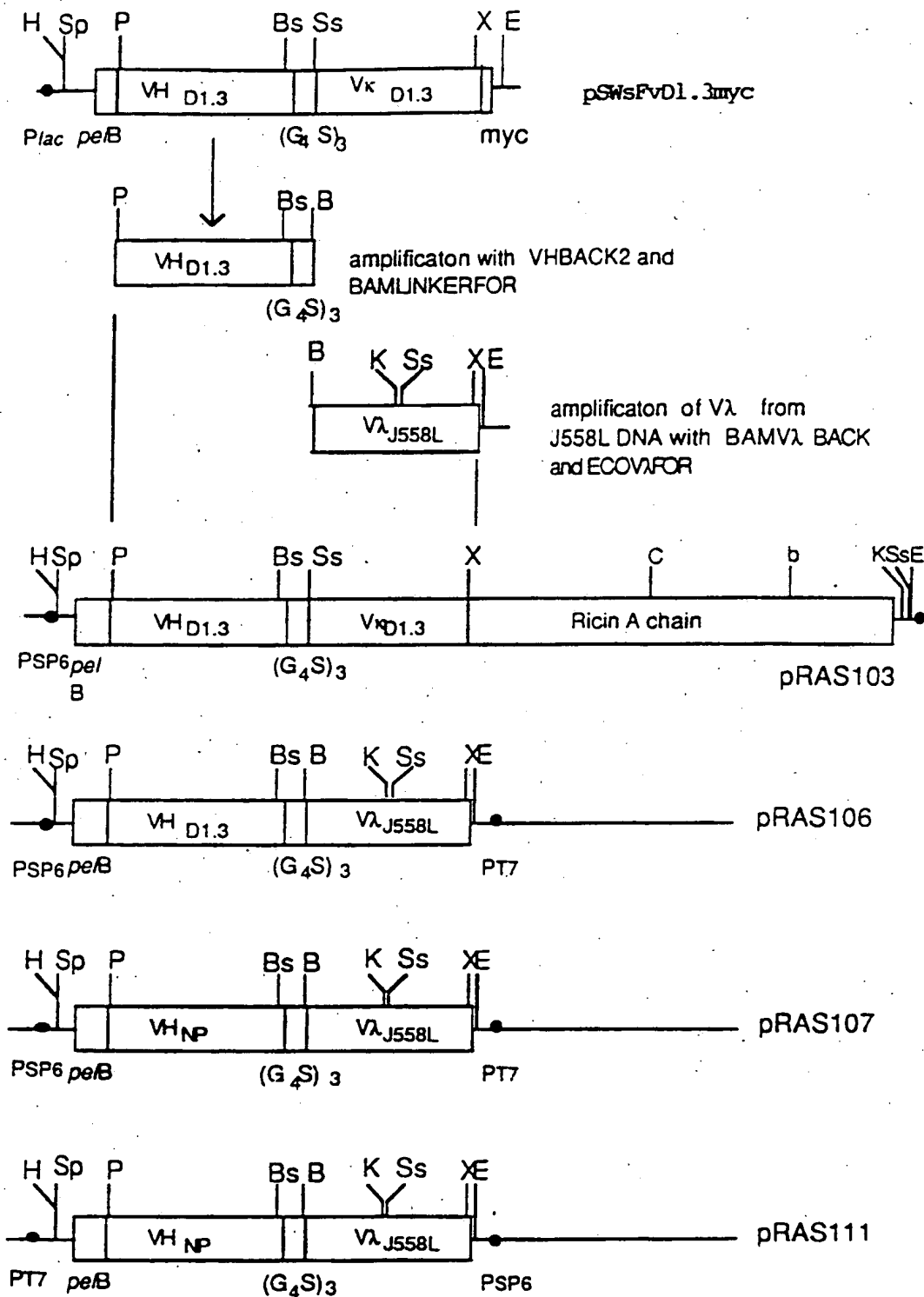


FIGURE 2

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VHBACK2	L Q E S G P CAGGTGCAGCTGCAGGAGTCAGGACC VHBACK2 <i>Pst</i> I
BAMLINKERFOR	G S GGATCCGACATCGAGCTCACTCAGTCTCCA <i>Bam</i> HI
BAMV\BACK	G S Q A E L T Q E S AAGCTTGGATCCCAGGCTGTTGTGACTCAGGAATCT <i>Bam</i> HI
ECOV\FOR	K L T V L G R S * * CCAAAGTACTGTCCTAGGTCTCGAGTAATAAGAATTCATGC <i>Xho</i> I <i>Eco</i> RI
VHBACK3	L Q Q P G CAGGTCCAAGTGCAGCAGCCTGG <i>Pst</i> I
VH1FOR-2	G Q G T T L T GGGGCCAAGGGACCACGGTCACCGTCTCCTCA <i>Bst</i> EII

FIGURE 3

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30

10 20 40 50

M K Y L L P

AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCT
HindIII SphI SD /-----

60 70 80 90 100 110

T A A A G L L L L A A Q P A M A Q V Q
ACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCAGGTGCAG
-----pelB leader-----/VHNP

120 130 140 150 160 170

L Q Q P G A E L V K P G A S V K L S C
CTGCAGCAGCCTGGGGCTGAGCTTGTGAAGCCTGGGGCTTCAGTGAAGCTGTCCTGC
PstI

180 190 200 210 220 2

K A S G Y T F T S Y W M H W V K Q R P
AAGGCTTCTGGCTACACCTTCACCAGCTACTGGATGCACTGGGTGAAGCAGAGGCCT
CDR1

30 240 250 260 270 280

G R G L E W I G R I D P N S G G T R Y
GGACGAGGCCTTGAGTGGATTGGAAGGATTGATCCTAATAGTGGTGGTACTAAGTAC
CDR2

290 300 310 320 330 340

N E K F L S K A T L T V D K P S S T A
AATGAGAAGTTCAAGAGCAAGGCCACACTGACTGTAGACAAACCCTCCAGCACAGCC

350 360 370 380 390 40

Y M Q L S S L T S E D S A V Y Y C A R
TACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTATTGTGCAAGA

0 410 420 430 440 450

Y D Y Y G S S Y F D Y W G Q G T T L T
TACGATTACTACGGTAGTAGCTACTTTGACTACTGGGGCCAAGGGACCACGGTCACC
CDR3 BstEII

460 470 480 490 500 510

V S S G G G G S G G G G S G G G G S Q
GTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCCAG
/------(G₄S)₃ linker-----BamHI-/Vλ

520 530 540 550 560 570

V V L T Q E S A L T T S P G E T V T L
GCTGTTGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAACAGTCACACTC

580 590 600 610 620

T C R S S T G A V T T S N Y A N W V Q
ACTTGTCTGCTCAAGTACTGGGGCTGTTACAAGTACTAGTCACTATGCCAAGTGGGTCCAA
CDR1

FIGURE 4 (START)

630 640 650 660 670 680
E K P D H L F T G L I G G T N N R A P
GAAAAACCAGATCATTATTCTACTGGTCTAATAGGTGGTACCAACAACCGAGCTCCA
KpnI CDR2 SstI

690 700 710 720 730 740
G V P A R F S G S L I G D K A A L T I
GGTGTTCCTGCCAGATTCTCAGGCTCCCTGATTGGAGACAAGGCTGCCCTCACCATC

750 760 770 780 790 8
T G A Q T E D E A I Y F C A L W Y S N
ACAGGGGCACAGACTGAGGATGAGGCAATATATTTCTGTGCTCTATGGTACAGCAAC
CDR3

00 810 820 830 840 850
H W V F G G G T K L T V L G L E * *
CACTGGGTGTTTCGGTGGAGGAACCAAAGTACTGTCCTAGGTCTCGAGTAATAAGAA
XhoI Eco

TTC
RI

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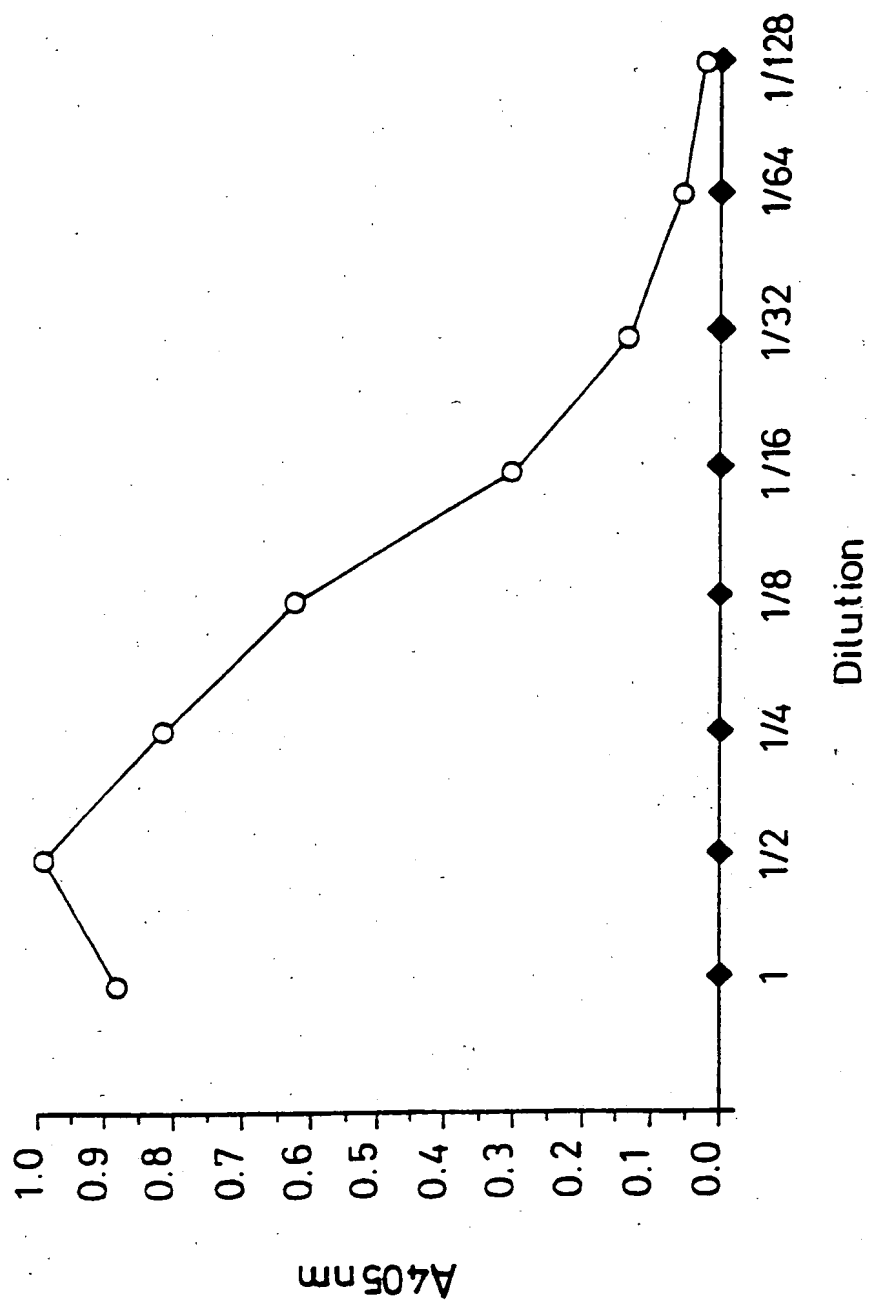


Fig. 5

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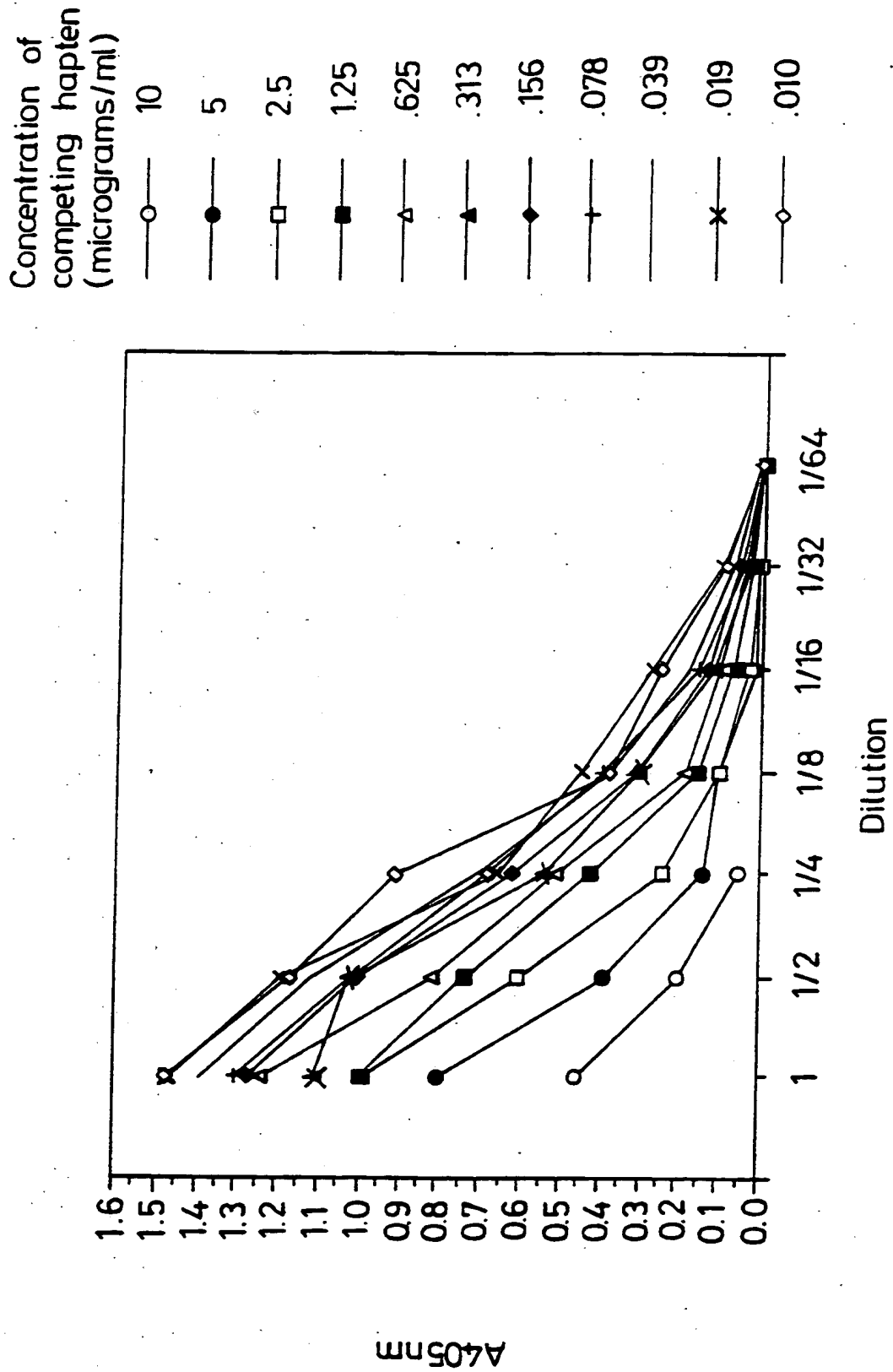


Fig. 6

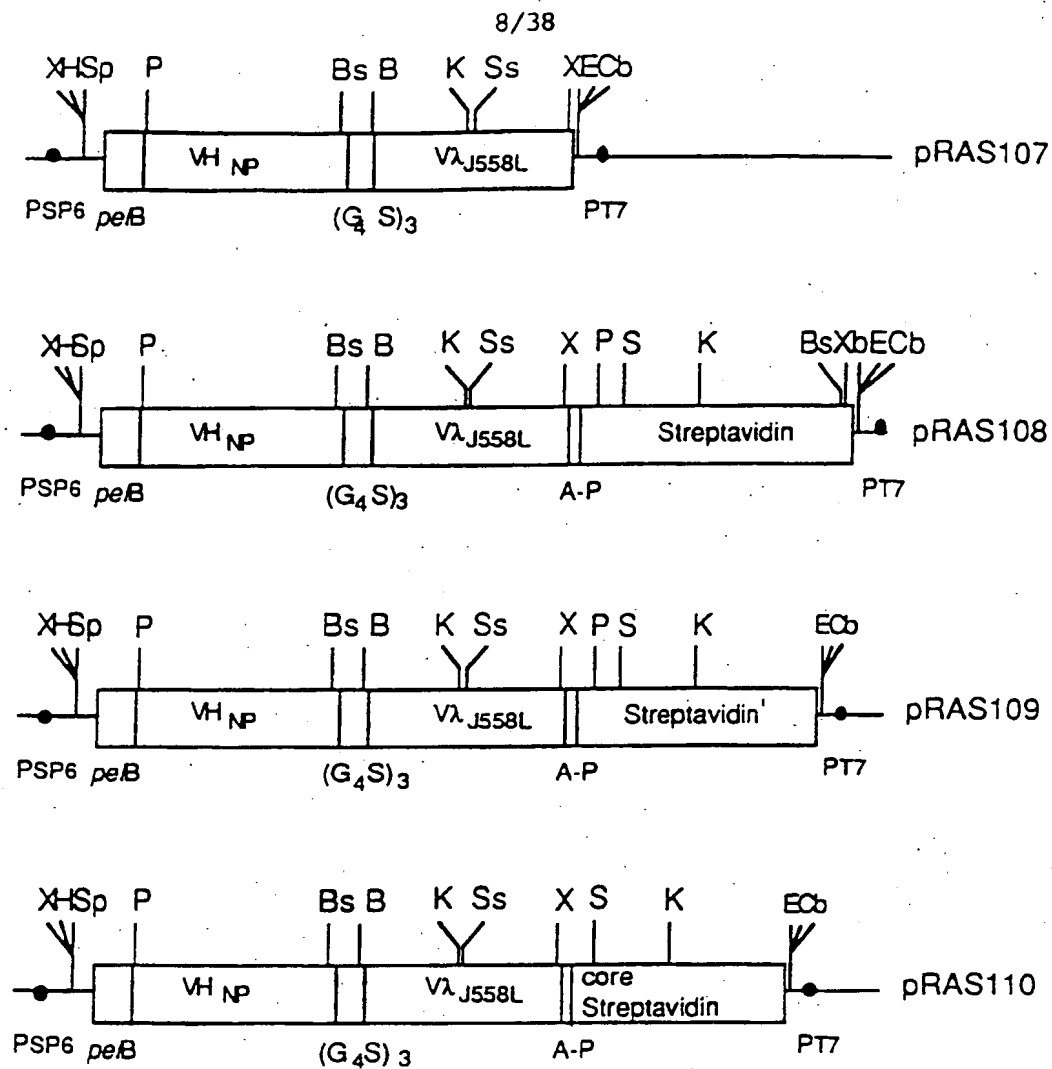


FIGURE 7

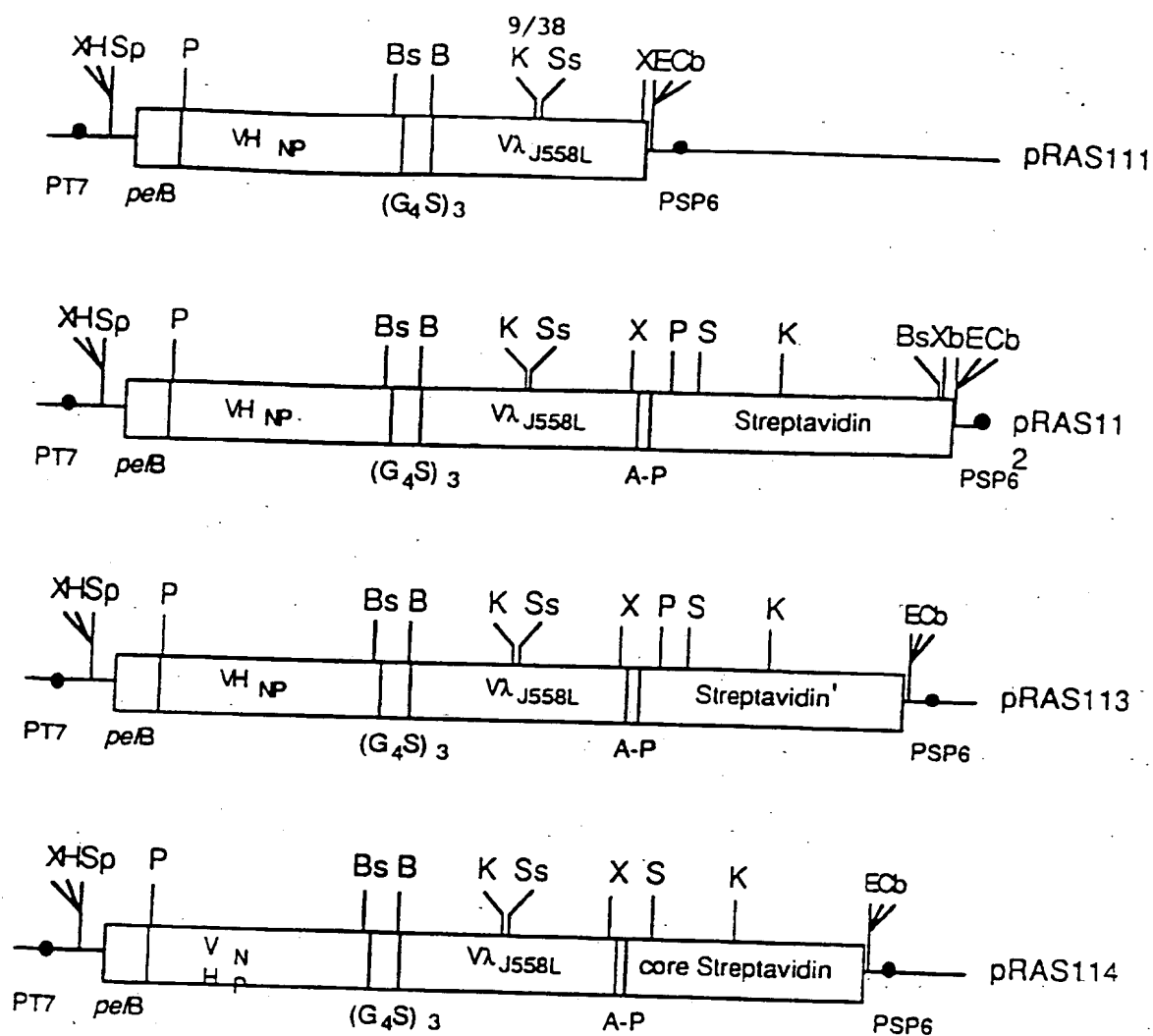


FIGURE 8

FIGURE 9 (START)

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790 800 810 820 830 840
 A L W Y S N H W V F G G T K L T V L G
GCTCTATGGTACAGCAACCACTGGGTGTTCCGGTGGAGGAACCAAAGCTGACTGTCTAGGT
 CDR3

850 860 870 880 890 900
 L E A P A A A P A D P S K D S K A Q V S
CTCGAGGCACCTGCTGCCGCACCTGCAGACCCGTCCAAGGACTCCAAAGCTCAGGTTTCT
 XhoI /-Ala.Pro linker-----/Streptavidin Ps

910 920 930 940 950 960
 A A E A G I T G T W Y N Q L G S T F I V
GCAGCCGAAGCTGGTATCACTGGCACCTGGTATAACCAACTGGGGTCGACTTTTCATTGTG
 tI SalI

970 980 990 1000 1010 1020
 T A G A D G A L T G T Y E S A V G N A E
 ACCGCTGGTGCGGACGGAGCTCTGACTGGCACCTACGAATCTGCGGTTGGTAACGCAGAA

1030 1040 1050 1060 1070 1080
 S R Y V L T G R Y D S A P A T D G S G T
 TCCCGCTACGTACTGACTGGCCGTTATGACTCTGCACCTGCCACCGATGGCTCTGGTACC
 KpnI

1090 1100 1110 1120 1130 1140
 A L G W T V A W K N N Y R N A H S A T T
 GCTCTGGGCTGGACTGTGGCTTGAAAAACAATATCGTAATGCGCACAGCGCCACTACG

1150 1160 1170 1180 1190 1200
 W S G Q Y V G G A E A R I N T Q W L L T
 TGGTCTGGCCAATACGTTGGCGGTGCTGAGGCTCGTATCAACACTCAGTGGCTGTTAACA

1210 1220 1230 1240 1250 1260
 S G T T E A N A W K S T L V G H D T F T
 TCCGGCACTACCGAAGCGAATGCATGGAAATCGACACTAGTAGGTCATGACACCTTTACC

1270 1280 1290 1300 1310 1320
 K V K P S A A S I D A A K K A G V N N G
 AAAGTTAAGCCTTCTGCTGCTAGCATTGATGCTGCCAAGAAAGCAGGCGTAAACAACGGT
 Bst

1330 1340 1350 1360
 N P L D A V Q Q * *
AACCCCTCTAGACGCTGTTCAAGCAATAATAAGAATTC
 EII XbaI EcoRI

FIGURE 9 (END)

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```

      10      20      30      40      50      60
          M K Y L L P T
AAGCTTGCATGCAAATTCATTTCAGGAGACAGTCATAATGAAATACCTATTGCCTACG
HindIIISphI          SD          /-----
      70      80      90      100      110      120
A A A G L L L L A A Q P A M A Q V Q L Q
GCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCAGGTGCAGCTGCAG
-----pelB leader-----/VHNP PstI
      130      140      150      160      170      180
Q P G A E L V K P G A S V K L S C K A S
CAGCCTGGGGCTGAGCTTGTGAAGCCTGGGGCTTCAGTGAAGCTGTCCTGCAAGGCTTCT
      190      200      210      220      230      240
G Y T F T S Y W M H W V K Q R P G R G L
GGCTACACCTTCACCAGCTACTGGATGCACTGGGTGAAGCAGAGGCCTGGACGAGGCCTT
          CDR1
      250      260      270      280      290      300
E W I G R I D P N S G G T R Y N E K F L
GAGTGGATTGGAAGGATTGATCCTAATAGTGGTGGTACTAAGTACAATGAGAAGTTCAAG
          CDR2
      310      320      330      340      350      360
S K A T L T V D K P S S T A Y M Q L S S
AGCAAGGCCACACTGACTGTAGACAAACCTCCAGCACAGCCTACATGCAGCTCAGCAGC
      370      380      390      400      410      420
L T S E D S A V Y Y C A R Y D Y Y G S S
CTGACATCTGAGGACTCTGCGGTCTATTATTGTGCAAGATACGATTACTACGGTAGTAGC
      430      440      450      460      470      480
Y F D Y W G Q G T T L T V S S G G G S
TACTTTGACTACTGGGGCCAAGGGACCAGGTACCGTCTCCTCAGGTGGAGGCGGTTCA
          CDR3          BstEII          /---(G4S)3---
      490      500      510      520      530      540
G G G G S G G G G S Q V V L T Q E S A L
GGCGGAGGTGGCTCTGGCGGTGGCGGATCCAGGCTGTTGTGACTCAGGAATCTGCACTC
-----BamHI- /VλJ558L
      550      560      570      580      590      600
T T S P G E T V T L T C R S S T G A V T
ACCACATCACCTGGTGAAACAGTCACACTCACTTGTGCTCAAGTACTGGGGCTGTTACA
          CDR1
      610      620      630      640      650      660
T S N Y A N W V Q E K P D H L F T G L I
ACTAGTAACTATGCCAACTGGGTCCAAGAAAAACCAGATCATTATTCACTGGTCTAATA
      670      680      690      700      710      720
G G T N N R A P G V P A R F S G S L I G
GGTGGTACCACAACCGAGCTCCAGGTGTTCTGCTCCAGATTCTCAGGCTCCCTGATTGGA
          KpnI          CDR2          SstI
      730      740      750      760      770      780
D K A A L T I T G A Q T E D E A I Y F C
GACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAGGCAATATATTTCTGT

```

FIGURE 10 (START)

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790 800 810 820 830 840
A L W Y S N H W V F G G G T K L T V L G
GCTCTATGGTACAGCAACCACTGGGTGTTCCGGTGGAGGAACCAAACTGACTGTCCTAGGT
CDR3

850 860 870 880 890 900
L E A P A A A P A D P S K D S K A Q V S
CTCGAGGCACCTGCTGCCGCACCTGCAGACCCGTCCTCAAGGACTCCAAAGCTCAGGTTTCT
XhoI /-Ala.Pro linker-----/Streptavidin Ps

910 920 930 940 950 960
A A E A G I T G T W Y N Q L G S T F I V
GCAGCCGAAGCTGGTATCACTGGCACCTGGTATAACCAACTGGGGTTCGACTTTTCATTGTG
tI SalI

970 980 990 1000 1010 1020
T A G A D G A L T G T Y E S A V G N A E
ACCGCTGGTGCGGACGGAGCTCTGACTGGCACCTACGAATCTGCGGTTGGTAACGCAGAA

1030 1040 1050 1060 1070 1080
S R Y V L T G R Y D S A P A T D G S G T
TCCCGCTACGTACTGACTGGCCGTTATGACTCTGCACCTGCCACCGATGGCTCTGGTACC
KpnI

1090 1100 1110 1120 1130 1140
A L G W T V A W K N N Y R N A H S A T T
GCTCTGGGCTGGACTGTGGCTTGGA AAAACA ACTATCGTAATGCGCACAGCGCCACTACG

1150 1160 1170 1180 1190 1200
W S G Q Y V G G A E A R I N T Q W L L T
TGGTCTGGCCAATACGTTGGCGGTGCTGAGGCTCGTATCAACACTCAGTGGCTGTTAACA

1210 1220 1230 1240 1250 1260
S G T T E A N A W K S T L V G H D T F T
TCCGGCACTACCGAAGCGAATGCATGGAAATCGACACTAGTAGGTCATGACACCTTTACC

1270 1280 1290
K V K P S A A S * *
AAAGTTAAGCCTTCTGCTGCTAGCTAATAAGAATTC
EcoRI

FIGURE 10 (END)

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```

      10      20      30      40      50      60
                        M K Y L L P T
AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACG
HindIIISphI                      SD /-----
      70      80      90     100     110     120
A A A G L L L L A A Q P A M A Q V Q L Q
GCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCAGGTGCAGCTGCAG
-----pelB leader-----/VHnp PstI

      130     140     150     160     170     180
Q P G A E L V K P G A S V K L S C K A S
CAGCCTGGGGCTGAGCTTGTGAAGCCTGGGGCTTCAGTGAAGCTGTCCTGCAAGGCTTCT

      190     200     210     220     230     240
G Y T F T S Y W M H W V K Q R P G R G L
GGCTACACCTTCACCAGCTACTGGATGCACTGGGTGAAGCAGAGGCCTGGACGAGGCCTT
CDR1

      250     260     270     280     290     300
E W I G R I D P N S G G T R Y N E K F L
GAGTGGATTGGAAGGATTGATCCTAATAGTGGTGGTACTAAGTACAATGAGAAGTTCAAG
CDR2

      310     320     330     340     350     360
S K A T L T V D K P S S T A Y M Q L S S
AGCAAGGCCACACTGACTGTAGACAAACCCTCCAGCACAGCCTACATGCAGCTCAGCAGC

      370     380     390     400     410     420
L T S E D S A V Y Y C A R Y D Y Y G S S
CTGACATCTGAGGACTCTGCGGTCTATTATTGTGCAAGATACGATTACTACGGTAGTAGC

      430     440     450     460     470     480
Y F D Y W G Q G T T L T V S S G G G S
TACTTTGACTACTGGGGCCAAGGGACCAGGTCTCCTCAGGTGGAGGCGGTTC
CDR3                      BstEII /---(G4S)3-----

      490     500     510     520     530     540
G G G G S G G G G S Q V V L T Q E S A L
GGCGGAGGTGGCTCTGGCGGTGGCGGATCCAGGCTGTTGTGACTCAGGAATCTGCACTC
-----BamHI-/VλJ558L

      550     560     570     580     590     600
T T S P G E T V T L T C R S S T G A V T
ACCACATCACCTGGTGAAACAGTCACACTCACTTGTGCTCAAGTACTGGGGCTGTTACA
CDR1

      610     620     630     640     650     660
T S N Y A N W V Q E K P D H L F T G L I
ACTAGTAAGTATGCCAACTGGGTCCAAGAAAAACCAGATCATTATTCACTGGTCTAATA

      670     680     690     700     710     720
G G T N N R A P G V P A R F S G S L I G
GGTGGTACCAACAACCGAGCTCCAGGTGTTCTGCTGCCAGATTCTCAGGCTCCCTGATTGGA
KpnI CDR2 SstI

      730     740     750     760     770     780
D K A A L T I T G A Q T E D E A I Y F C
GACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAGGCAATATATTTCTGT

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FIGURE 11 (START)
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790 800 810 820 830 840
A L W Y S N H W V F G G G T K L T V L G
GCTCTATGGTACAGCAACCACTGGGTGTTCCGGTGGAGGAACCAAACTGACTGTCCTAGGT
CDR3

850 860 870 880 890 900
L E A P A A A P A E A G I T G T W Y N Q
CTCGAGGCACCTGCTGCCGCACCTGCCGAAGCTGGTATCACTGGCACCTGGTATAACCAA
XhoI /-Ala.Pro linker--/core streptavidin

910 920 930 940 950 960
L G S T F I V T A G A D G A L T G T Y E
CTGGGGTCGACTTTTCATTGTGACCGCTGGTGCGGACGGAGCTCTGACTGGCACCTACGAA
SalI

970 980 990 1000 1010 1020
S A V G N A E S R Y V L T G R Y D S A P
TCTGCGGTTGGTAACGCAGAATCCCGCTACGTACTGACTGGCCGTTATGACTCTGCACCT

1030 1040 1050 1060 1070 1080
A T D G S G T A L G W T V A W K N N Y R
GCCACCGATGGCTCTGGTACCGCTCTGGGCTGGACTGTGGCTTGGAAAAACAACTATCGT
KpnI

1090 1100 1110 1120 1130 1140
N A H S A T T W S G Q Y V G G A E A R I
AATGCGCACAGCGCCACTACGTGGTCTGGCCAATACGTTGGCGGTGCTGAGGCTCGTATC

1150 1160 1170 1180 1190 1200
N T Q W L L T S G T T E A N A W K S T L
AACACTCAGTGGCTGTTAACATCCGGCACTACCGAAGCGAATGCATGGAAATCGACACTA

1210 1220 1230 1240 1250 1260
V G H D T F T K V K P S A A S * *
GTAGGTCATGACACCTTTACCAAAGTTAAGCCTTCTGCTGCTAGCTAATAAGAATTC
EcoRI

FIGURE 11 (END)

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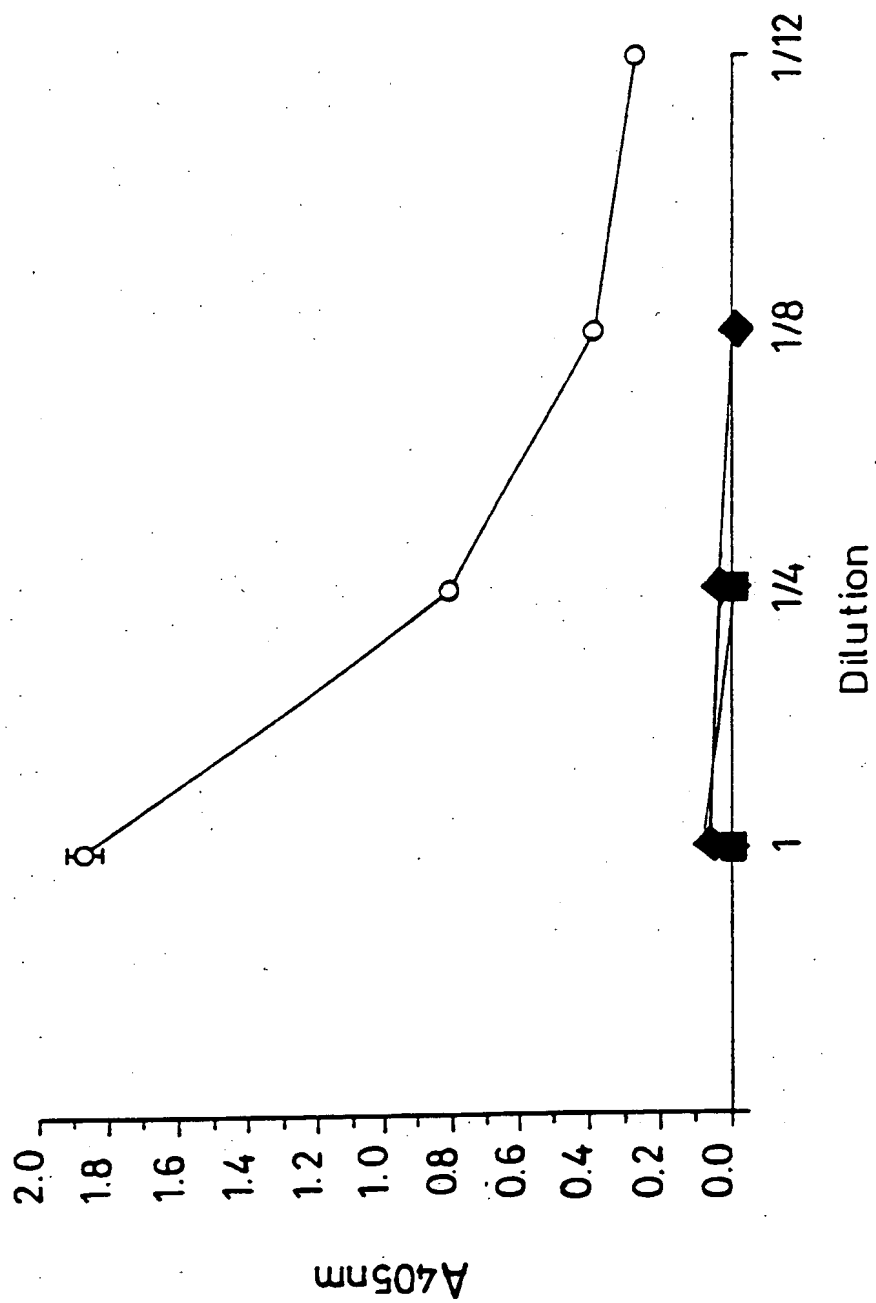


Fig. 12

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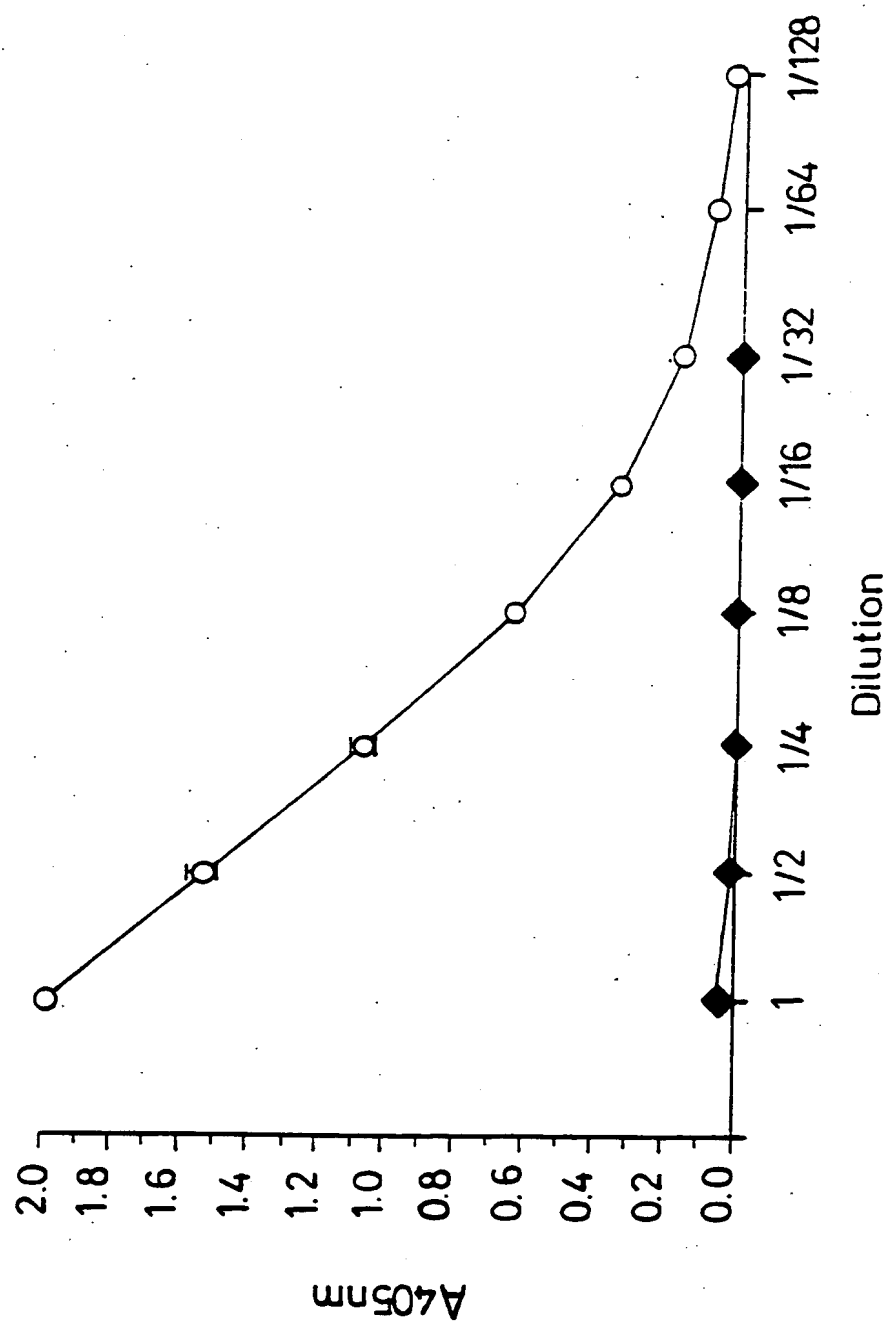


Fig. 13

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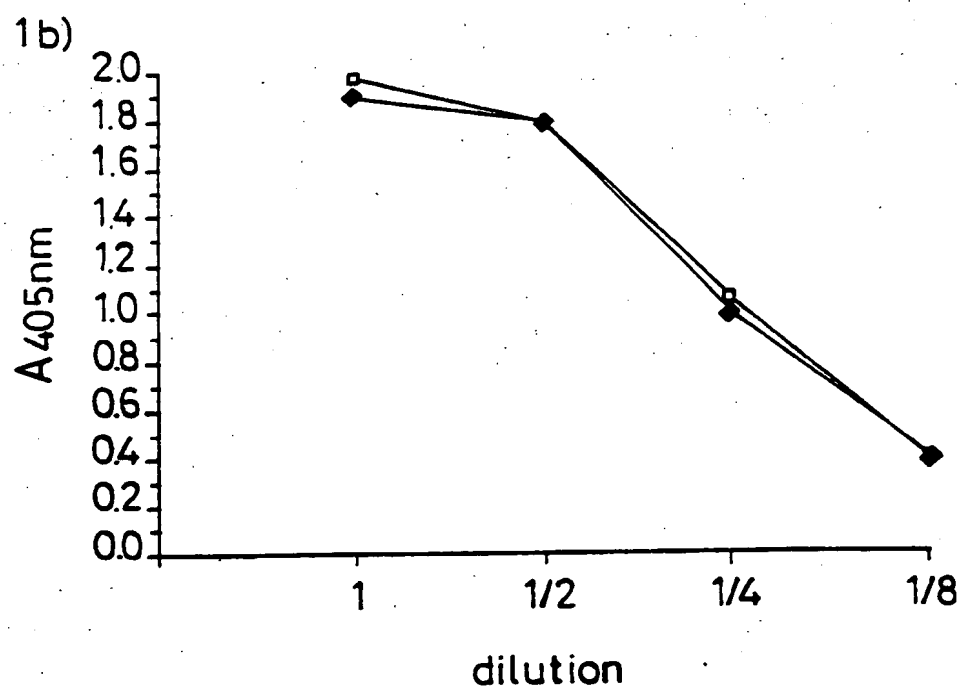
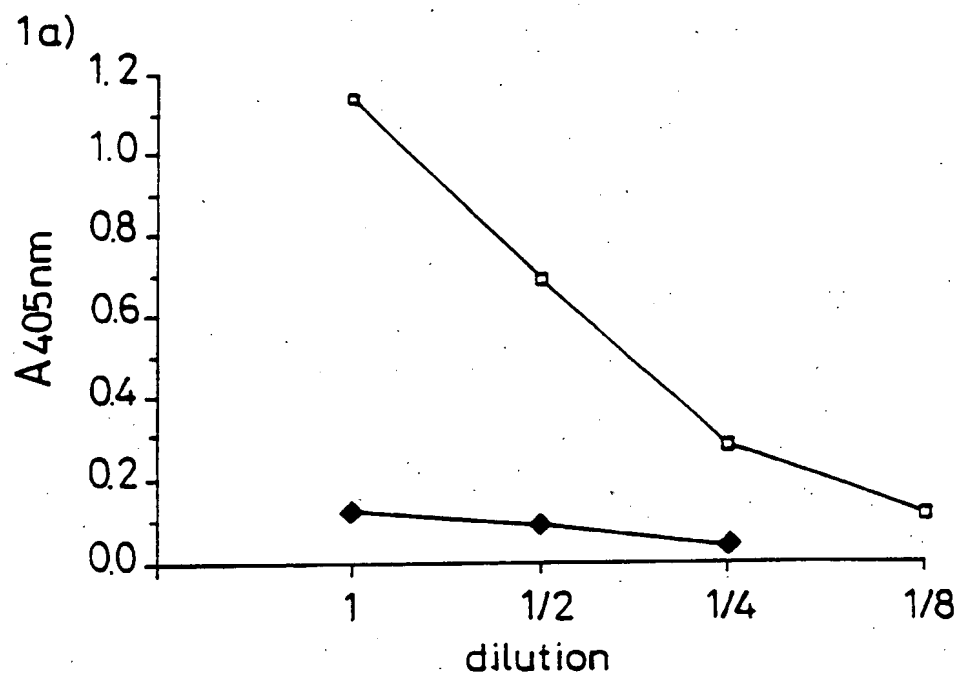


Fig. 14

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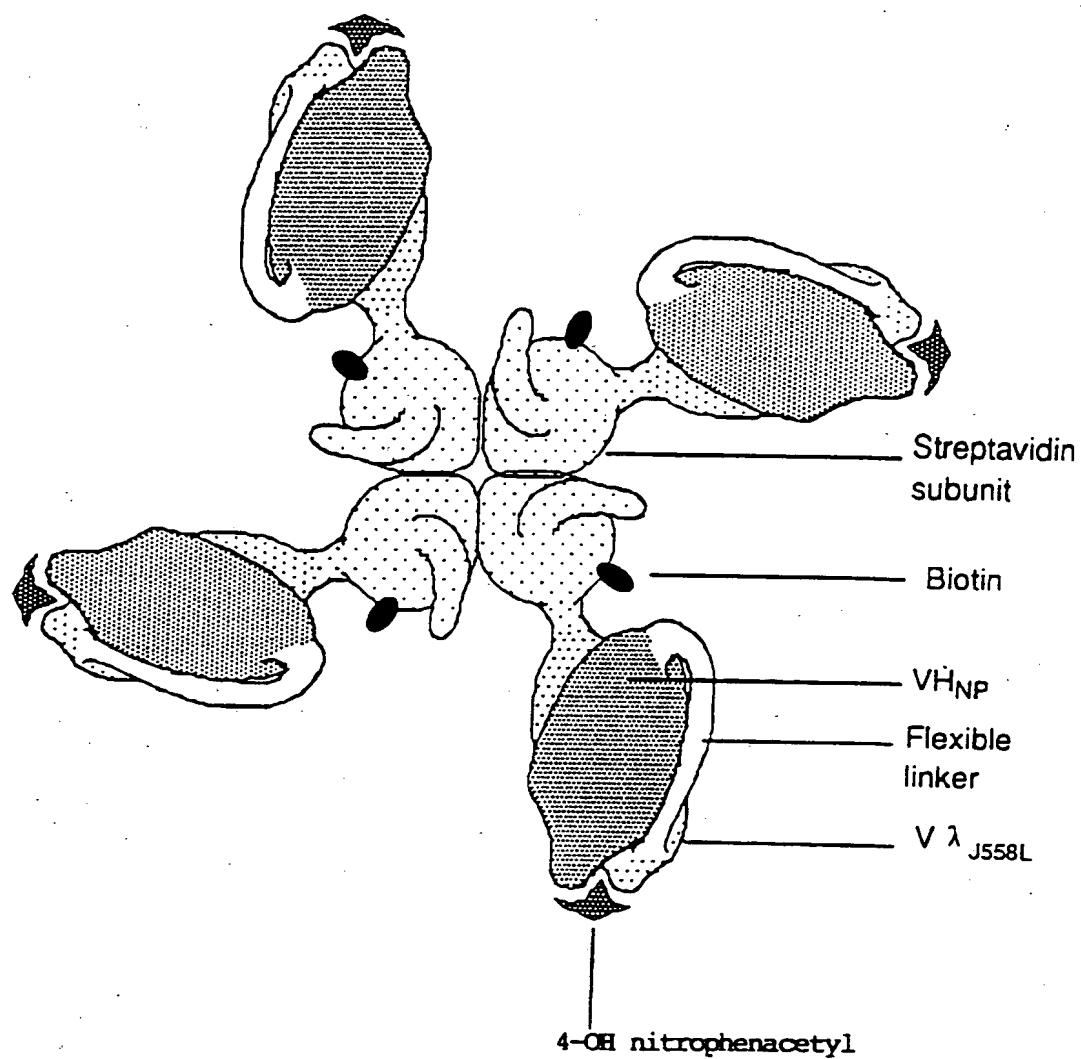


FIGURE 15

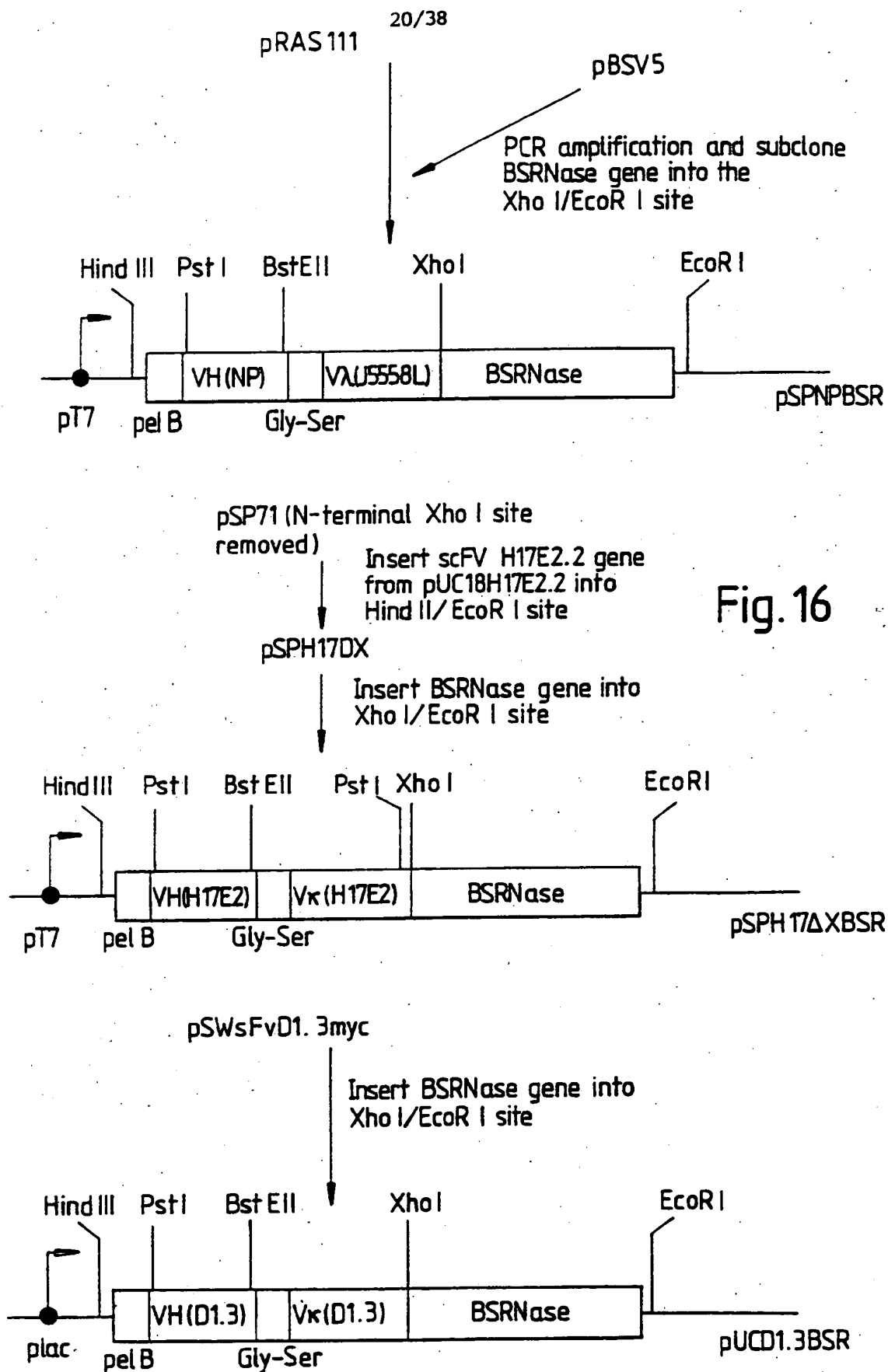


Fig. 16

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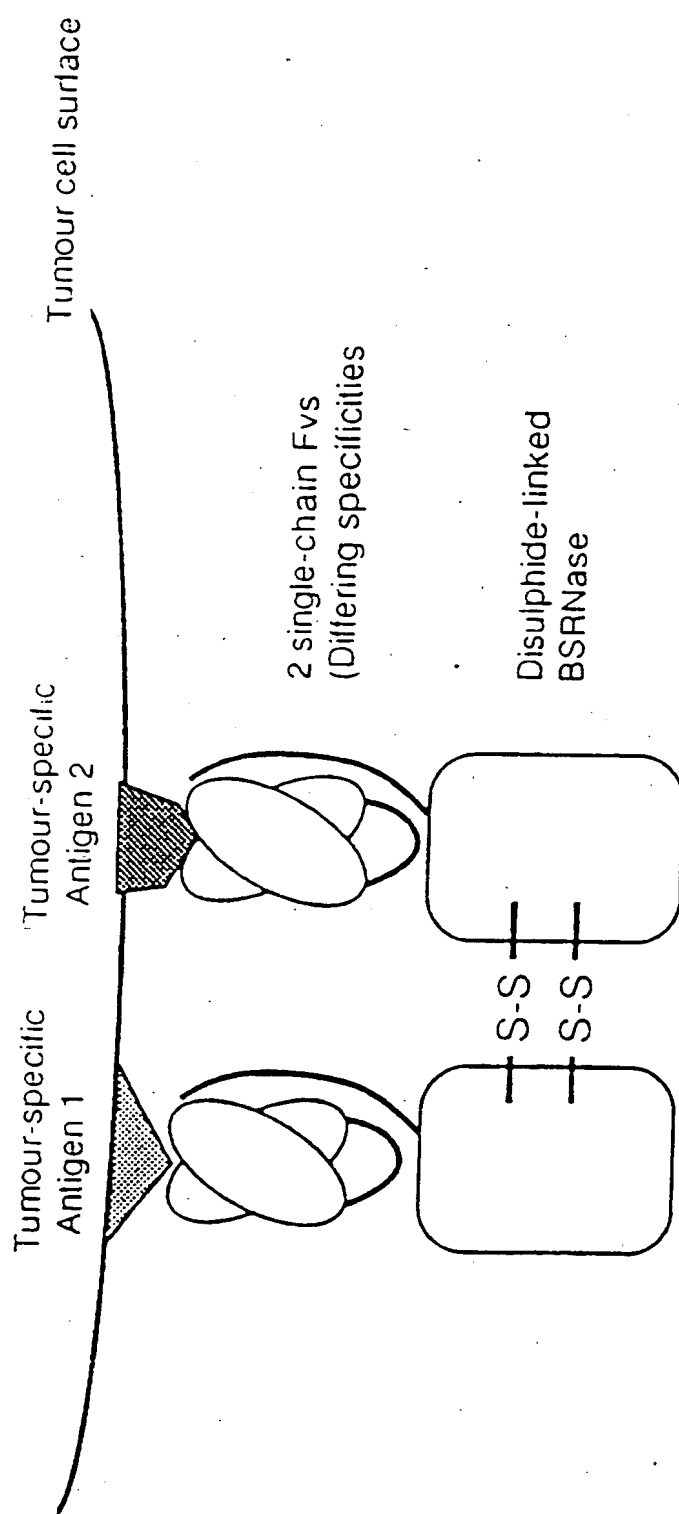


FIGURE 17

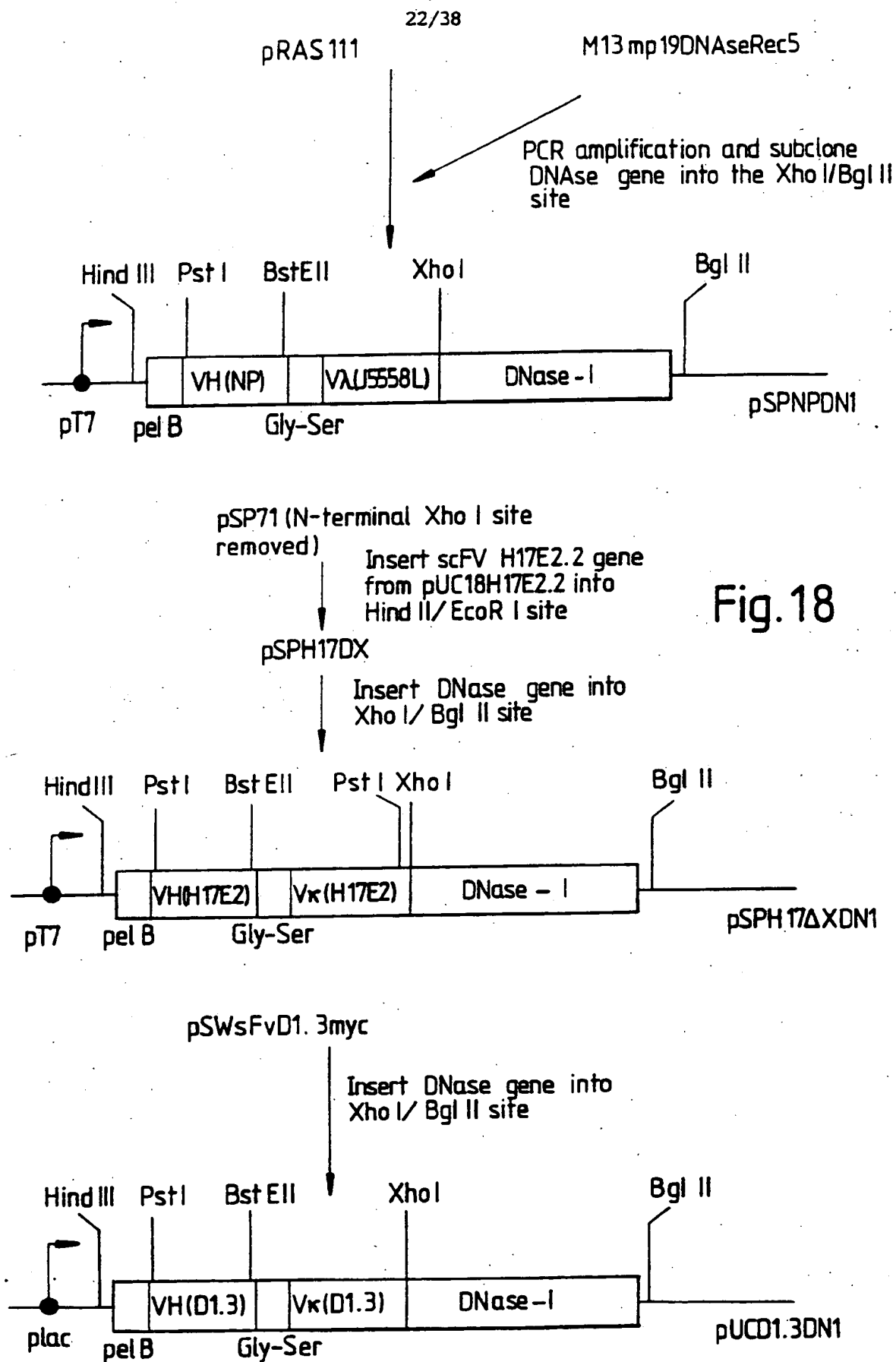


Fig.18

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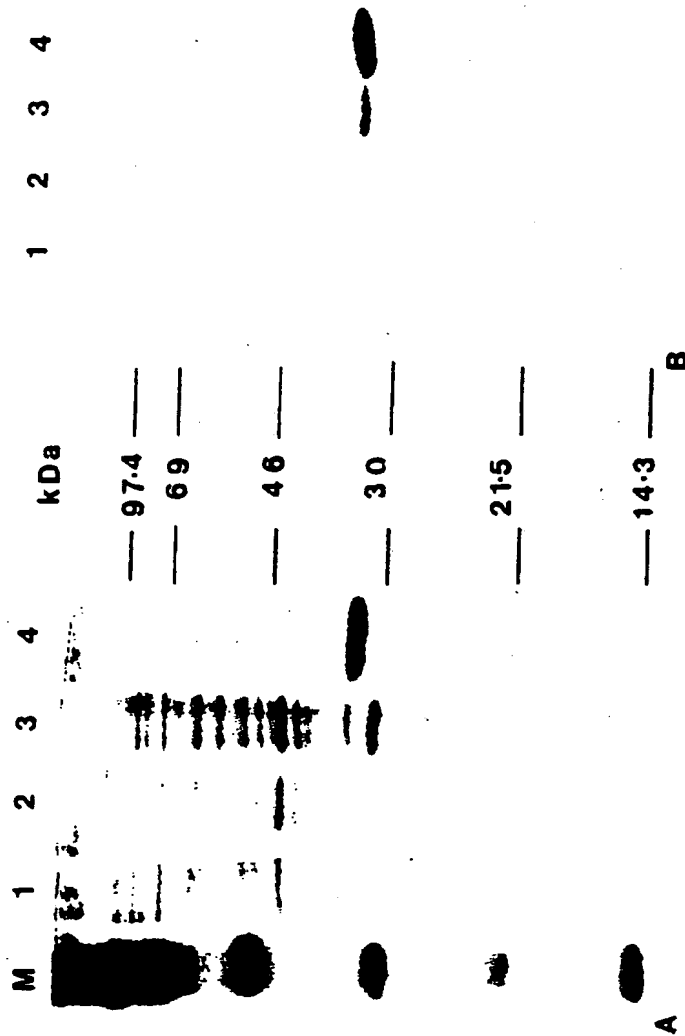


Fig.19

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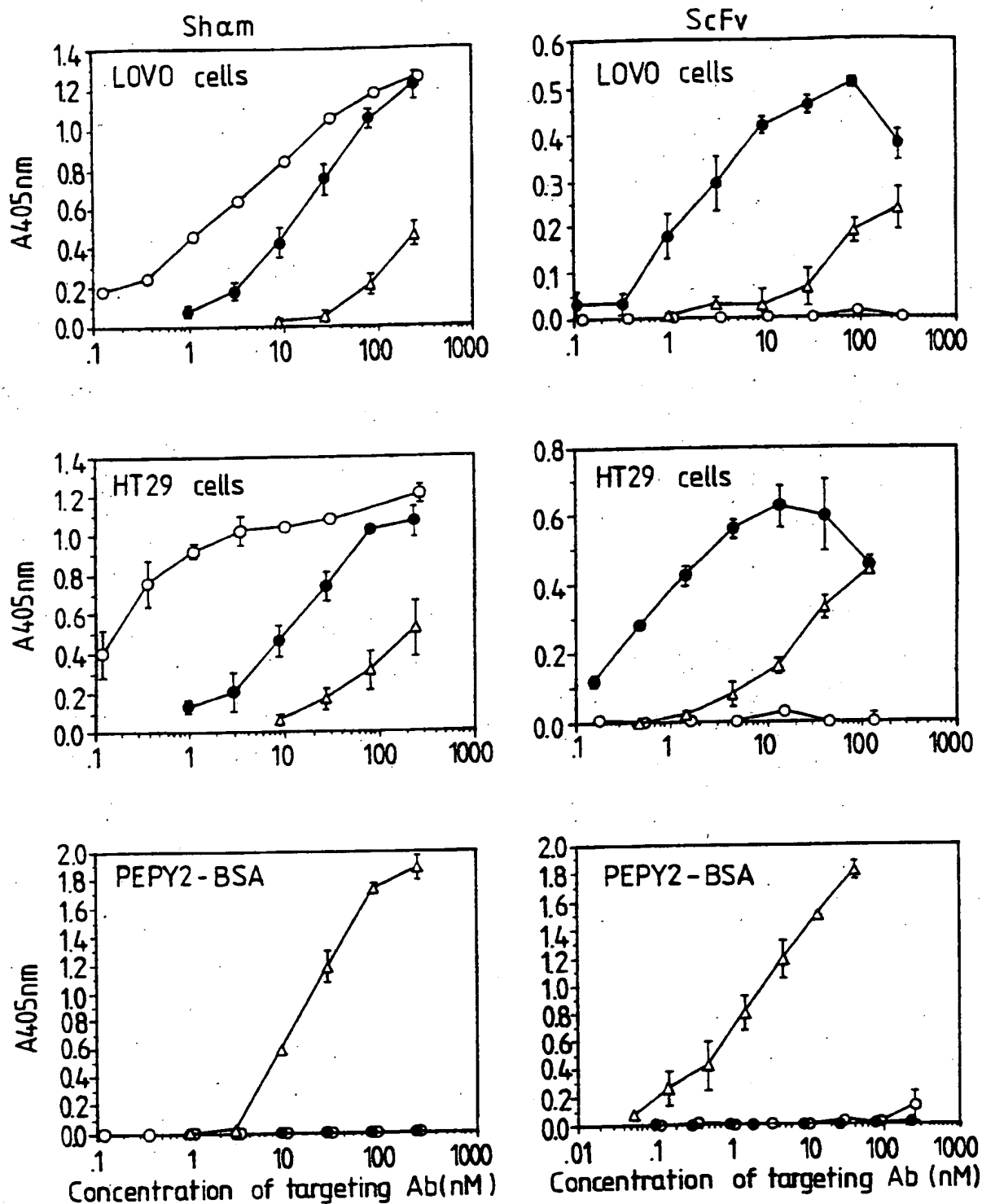


Fig. 20

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AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTA
TTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCCAACCAGCGATG
GCCCAGGTGCAGCTGCAGCAGCCTGGGGCTGAGCTTGTGAAGCCTGGGGCT
TCAGTGAAGCTGTCCTGCAAGGCTTCTGGCTACACCTTCACCAGCTACTGG
ATGCACTGGGTGAAGCAGAGGCCTGGACGAGGCCTTGAGTGGATTGGAAGG
ATTGATCCTAATAGTGGTGGTACTAAGTACAATGAGAAGTTCAAGAGCAAG
GCCACACTGACTGTAGACAAACCCTCCAGCACAGCCTACATGCAGCTCAGC
AGCCTGACATCTGAGGACTCTGCGGTCTATTATTGTGCAAGATACGATTAC
TACGGTAGTAGCTACTTTGACTACTGGGGCCAAGGGACCACGGTCACCGTC
TCCTCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCC
CAGGCTGTTGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAACA
GTCACACTCACTTGTCGCTCAAGTACTGGGGCTGTTACAACTAGTAACTAT
GCCAACTGGGTCCAAGAAAAACCAGATCATTATTTACTGGTCTAATAGGT
GGTACCAACAACCGAGCTCCAGGTGTTCTGCCAGATTCTCAGGCTCCCTG
ATTGGAGACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAG
GCAATATATTTCTGTGCTCTATGGTACAGCAACCACTGGGTGTTCCGGTGGA
GGAACCAAACTGACTGTCCTAGGTCTCGAGATCAAGCGCAAGGAATCTGCA
GCTGCCAAGTTCGAGCGGCAGCACATGGACTCTGGCAACTCCCCCAGCAGC
AGCTCCAACTACTGCAACCTGATGATGTGCTGCCGAAGATGACCCAGGGGA
AATGCAAGCCAGTGAACACCTTTGTGCATGAGTCCCTGGCCGATGTTAAGG
CCGTGTGCTCCCAGAAGAAAGTCACTTGCAAGAATGGGCAGACCAACTGCT
ACCAGAGCAAATCCACCATGCGCATCACAGACTGCCGCGAGACTGGCAGCT
CCAAGTACCCCAACTGCGCCTACAAGACCACCCAGGTGGAGAAACACATCA
TAGTGGCTTGTGGCGGTAAACCGTCCGTGCCAGTCCACTTCGATGCTTCAG
TG TAGATCTCCACCTGAGGCCAGAACAGTGAATTC

FIGURE 21

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AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTA
TTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATG
GCCCAGGTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTG
TCCATCACATGCACTGTCTCAGGGTTCTCATTAAACCAGTTATGGTGTAAAGC
TGGGTTCGCCAGCCTCCAAGAAAGGGTCTGGAGTGGCTGGGAGTAATATGG
GAAGACGGGAGCACAAATTATCATTACAGTCTCATATCCAGACTGAGCATC
AACAAGGATAACTCCAAGAGCCAAGTTTTCTTAAACTGAACAGTCTGCAA
ACTGATGACACAGCCAGTACTACTGTGCCAAACCCCACTACGGTAGCAGC
AACGTGGGGGCTATGGAATACTGGGGTCAAGGAACCTCGGTACCGTCTCC
TCAGGTGGAGGCGGTTCAAGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAC
ATCGAGCTCACCCAGTCTCCAGCCTCCCTAACTGCATCTGTGGGAGAACT
GTCACCATCACCTGTGAGCAAGTGAAAATATTTACAGTTATGTAGCATGG
TATCAGCAGAAACAGGGAAAATCTCCTCAGTTCCTGGTCTATAATGCAAAA
TCCTTAGCAGAGGGTGTGCCATCAAGGTTCAAGTGGCAGTGGATCAGGCACA
CAGTTTTCTCTGAAGATCAACAGCCTGCAGCCTGAAAATTTTGGGAATTAT
TACTGTCAACATCATTATGTTAGTCCGTGGACGTTCCGGTGGAGGCACCAAG
CTCGAGATCAAGCGCAAGGAATCTGCAGCTGCCAAGTTCGAGCGGCAGCAC
ATGGACTCTGGCAACTCCCCCAGCAGCAGCTCCAACTACTGCAACCTGATG
ATGTGCTGCCGAAGATGACCCAGGGGAAATGCAAGCCAGTGAACACCTTTG
TGCATGAGTCCCTGGCCGATGTTAAGGCCGTGTGCTCCCAGAAGAAAGTCA
CTTGCAAGAATGGGCAGACCAACTGCTACCAGAGCAAATCCACCATGCGCA
TCACAGACTGCCGCGAGACTGGCAGCTCCAAGTACCCCAACTGCGCCTACA
AGACCACCCAGGTGGAGAAACACATCATAGTGGCTTGTGGCGGTAAACCGT
CCGTGCCAGTCCACTTCGATGCTTCAGTGTAGATCTCCACCTGAGGCCAGA
ACAGTGAATTC

FIGURE 22

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AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTA
TTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCCAACCAGCGATG
GCCCAGGTGCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAG
ACGCTGTCCATCACATGCACCGTCTCAGGGTTCTCATTAACCGGCTATGGT
GTAAACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAATG
ATTTGGGGTGATGGAAACACAGACTATAATTCAGCTCTCAAATCCAGACTG
AGCATCAGCAAGGACAACCTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGT
CTGCACACTGATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTAT
AGGCTTGACTACTGGGGCCAAGGCACCACGGTCACCGTCTCCTCAGGTGGA
GGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCGTCATG
ACTCAGTCTCCAGCCTCCCTTTCTGCGTCTGTGGGAGAACTGTCAACCATC
ACATGTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTATCAGCAG
AAACAGGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAACCTTAGCA
GATGGTGTGCCATCAAGGTTTCAGTGGCAGTGGATCAGGAACACAATATTCT
CTCAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAA
CATTTTTGGAGTACTCCTCGGACGTTTCGGTGGAGGCACCAAGCTCGAGATC
AAGCGCAAGGAATCTGCAGCTGCCAAGTTCGAGCGGCAGCACATGGACTCT
GGCAACTCCCCCAGCAGCAGCTCCAAGTACTGCAACCTGATGATGTGCTGC
CGAAGATGACCCAGGGGAAATGCAAGCCAGTGAACACCTTTGTGCATGAGT
CCCTGGCCGATGTTAAGGCCGTGTGCTCCCAGAAGAAAGTCACTTGCAAGA
ATGGGCAGACCAACTGCTACCAGAGCAAATCCACCATGCGCATCACAGACT
GCCGCGAGACTGGCAGCTCCAAGTACCCCAACTGCGCCTACAAGACCACCC
AGGTGGAGAAACACATCATAGTGGCTTGTGGCGGTAAACCGTCCGTGCCAG
TCCACTTCGATGCTTCAGTGTAGATCTCCACCTGAGGCCAGAACAGTGAAT
TC

FIGURE 23

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AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTA
TTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCCAACCAGCGATG
GCCCAGGTGCAGCTGCAGCAGCCTGGGGCTGAGCTTGTGAAGCCTGGGGCT
TCAGTGAAGCTGTCCTGCAAGGCTTCTGGCTACACCTTCACCAGCTACTGG
ATGCACTGGGTGAAGCAGAGGCCTGGACGAGGCCTTGAGTGGATTGGAAGG
ATTGATCCTAATAGTGGTGGTACTAAGTACAATGAGAAGTTCAAGAGCAAG
GCCACACTGACTGTAGACAAACCCTCCAGCACAGCCTACATGCAGCTCAGC
AGCCTGACATCTGAGGACTCTGCGGTCTATTATTGTGCAAGATACGATTAC
TACGGTAGTAGCTACTTTGACTACTGGGGCCAAGGGACCACCGTCACCGTC
TCCTCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCC
CAGGCTGTTGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAACA
GTCACACTCACTTGTGCTCAAGTACTGGGGCTGTTACAACCTAGTAACTAT
GCCAACTGGGTCCAAGAAAACCAGATCATTTATTCACTGGTCTAATAGGT
GGTACCAACAACCGAGCTCCAGGTGTTCTGCCAGATTCTCAGGCTCCCTG
ATTGGAGACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAG
GCAATATATTTCTGTGCTCTATGGTACAGCAACCACTGGGTGTTCCGGTGGA
GGAACCAAACCTGACTGTCCTAGGTCTCGAGATTAAACGTATGCTTAAGATC
GCTGCTTTCAACATACGTACCTTCGGTGAATCTAAAATGTCTAACGCTACG
CTAGCATCTTACATCGTACGCATCGTACGCCGTTACGATATCGTTCTGATC
CAGGAAGTTCGCGACTCTCACCTGGTTGCAGTTGGTAAACTTCTAGACTAC
CTGAACCAGGACGACCCGAACACCTACCACTACGTTGTTTCTGAACCCCTC
GGGCGTAACTCTTACAAAGAACGGTACCTGTTCTGTTCCGTCCGAACAAA
GTTTCAGTACTGGATACCTACCAGTACGACGACGGATGCGAATCTTGCGGT
AACGACTCTTTCTCCCGGGAACCGGCTGTTGTTAAATTCTCGAGCCACTCT
ACCAAGGTAAAGAGTTCGCTATCGTTGCTCTGCACAGCGCGCGCTCTGAC
GCTGTTGCTGAAATCAACTCTCTGTACGACGTTTACCTGGACGTTACAGCAG
AAATGGCACCTGAACGACGTCATGCTGATGGGTGACTTCAACGCTGACTGC
TCTTATGTAACCTCTTCTCAGTGGTCATCGATTCTGCTGCGCACCTCGTCG
ACCTTCCAGTGGCTGATCCCGGACTCCGCTGACACCACCGCTACTAGTACC
AACTGCGCTTACGACCGTATCGTTGTTGCTGGATCCCTGCTGCAGTCTTCT
GTTGTACCGGGTAGCGCGGCCCGTTCCAGGCTGCATATGGTCTT
TCGAACGAAATGGCGCTGGCCATCTCTGATCACTACCCGGTTGAGGTAACC
CTGACCTAATTCTAGA

FIGURE 24

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AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTA
TTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATG
GCCCAGGTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTG
TCCATCACATGCACTGTCTCAGGGTTCTCATTAAACCAGTTATGGTGTAAGC
TGGGTTTCGCCAGCCTCCAAGAAAGGGTCTGGAGTGGCTGGGAGTAATATGG
GAAGACGGGAGCACAAATTATCATTACGTCTCATATCCAGACTGAGCATC
AACAAGGATAACTCCAAGAGCCAAGTTTTCTTAAACTGAACAGTCTGCAA
ACTGATGACACAGCCACGTACTACTGTGCCAAACCCCACTACGGTAGCAGC
AACGTGGGGGCTATGGAATACTGGGGTCAAGGAACCTCGGTACCGTCTCC
TCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAC
ATCGAGCTCACCCAGTCTCCAGCCTCCCTAACTGCATCTGTGGGAGAACT
GTCACCATCACCTGTCGAGCAAGTGAAAATATTTACAGTTATGTAGCATGG
TATCAGCAGAAACAGGGGAAAATCTCCTCAGTTCCTGGTCTATAATGCAAAA
TCCTTAGCAGAGGGTGTGCCATCAAGGTTTCAGTGGCAGTGGATCAGGCACA
CAGTTTTCTCTGAAGATCAACAGCCTGCAGCCTGAAAATTTTGGGAATTAT
TACTGTCAACATCATTATGTTAGTCCGTGGACGTTCCGTGGAGGCACCAAG
CTCGAGATTAAACGTATGCTTAAGATCGCTGCTTTCAACATACGTACCTTC
GGTGAATCTAAAATGTCTAACGCTACGCTAGCATCTTACATCGTACGCATC
GTACGCCGTTACGATATCGTTCTGATCCAGGAAGTTCGCGACTCTCACCTG
GTTGCAGTTGGTAAACTTCTAGACTACCTGAACCAGGACGACCCGAACACC
TACCACTACGTTGTTTCTGAACCCCTCGGGCGTAACTCTTACAAAGAACGG
TACCTGTTCCCTGTTCCGTCCGAACAAAGTTTCAGTACTGGATACCTACCAG
TACGACGACGGATGCGAATCTTGCGGTAACGACTCTTTCTCCCGGGAACCG
GCTGTTGTTAAATTCTCGAGCCACTCTACCAAGGTTAAAGAGTTCGCTATC
GTTGCTCTGCACAGCGCGCCGTCTGACGCTGTTGCTGAAATCAACTCTCTG
TACGACGTTTACCTGGACGTTTCAGCAGAAATGGCACCTGAACGACGTCATG
CTGATGGGTGACTTCAACGCTGACTGCTCTTATGTAACCTCTTCTCAGTGG
TCATCGATTTCGTCTGCGCACCTCGTCGACCTTCCAGTGGCTGATCCCGGAC
TCCGCTGACACCACCGCTACTAGTACCAACTGCGCTTACGACCGTATCGTT
GTTGCTGGATCCCTGCTGCAGTCTTCTGTTGTACCGGGTAGCGCGGCCCGG
TTCGACTTCCAGGCTGCATATGGTCTTTTGAACGAAATGGCGCTGGCCATC
TCTGATCACTACCCGGTTGAGGTAACCTGACCTAATTCTAGA

FIGURE 25

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AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTA
TTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATG
GCCCAGGTGCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAG
ACGCTGTCCATCACATGCACCGTCTCAGGGTTCTCATTAAACCGGCTATGGT
GTAAACTGGGTTCCGCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAATG
ATTTGGGGTGATGGAAACACAGACTATAATTCAGCTCTCAAATCCAGACTG
AGCATCAGCAAGGACAACCTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGT
CTGCACACTGATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTAT
AGGCTTGACTACTGGGGCCAAGGCACCACGGTCACCGTCTCCTCAGGTGGA
GGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCGTCATG
ACTCAGTCTCCAGCCTCCCTTTCTGCGTCTGTGGGAGAACTGTCACCATC
ACATGTGCGAGCAAGTGGAATATTACAATTATTTAGCATGGTATCAGCAG
AAACAGGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAACCTTAGCA
GATGGTGTGCCATCAAGGTTCAAGTGGCAGTGGATCAGGAACACAATATTCT
CTCAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAA
CATTTTTGGAGTACTCCTCGGACGTTCCGGTGGAGGCACCAAGCTCGAGATT
AAACGTATGCTTAAGATCGCTGCTTTCAACATACGTACCTTCGGTGAATCT
AAAATGTCTAACGCTACGCTAGCATCTTACATCGTACGCATCGTACGCCGT
TACGATATCGTTCTGATCCAGGAAGTTCGCGACTCTCACCTGGTTGCAGTT
GGTAAACTTCTAGACTACCTGAACCAGGACGACCCGAACACCTACCACTAC
GTTGTTTCTGAACCCCTCGGGCGTAACCTCTTACAAAGAACGGTACCTGTTT
CTGTTCCGTCCGAACAAAGTTTCAGTACTGGATACCTACCAGTACGACGAC
GGATGCGAATCTTGCGGTAACGACTCTTTCTCCCGGAACCGGCTGTTGTT
AAATTCTCGAGCCACTCTACCAAGGTTAAAGAGTTCGCTATCGTTGCTCTG
CACAGCGCGCCGTCTGACGCTGTTGCTGAAATCAACTCTCTGTACGACGTT
TACCTGGACGTTTACGCAGAAATGGCACCTGAACGACGTCATGCTGATGGGT
GACTTCAACGCTGACTGCTCTTATGTAACCTCTTCTCAGTGGTTCATCGATT
CGTCTGCGCACCTCGTCGACCTTCCAGTGGCTGATCCCGGACTCCGCTGAC
ACCACCGCTACTAGTACCAACTGCGCTTACGACCGTATCGTTGTTGCTGGA
TCCCTGCTGCAGTCTTCTGTTGTACCGGGTAGCGCGGCCCCGTTTCGACTTC
CAGGCTGCATATGGTCTTTTCGAACGAAATGGCGCTGGCCATCTCTGATCAC
TACCCGGTTGAGGTAACCCTGACCTAATTCTAGA

FIGURE 26

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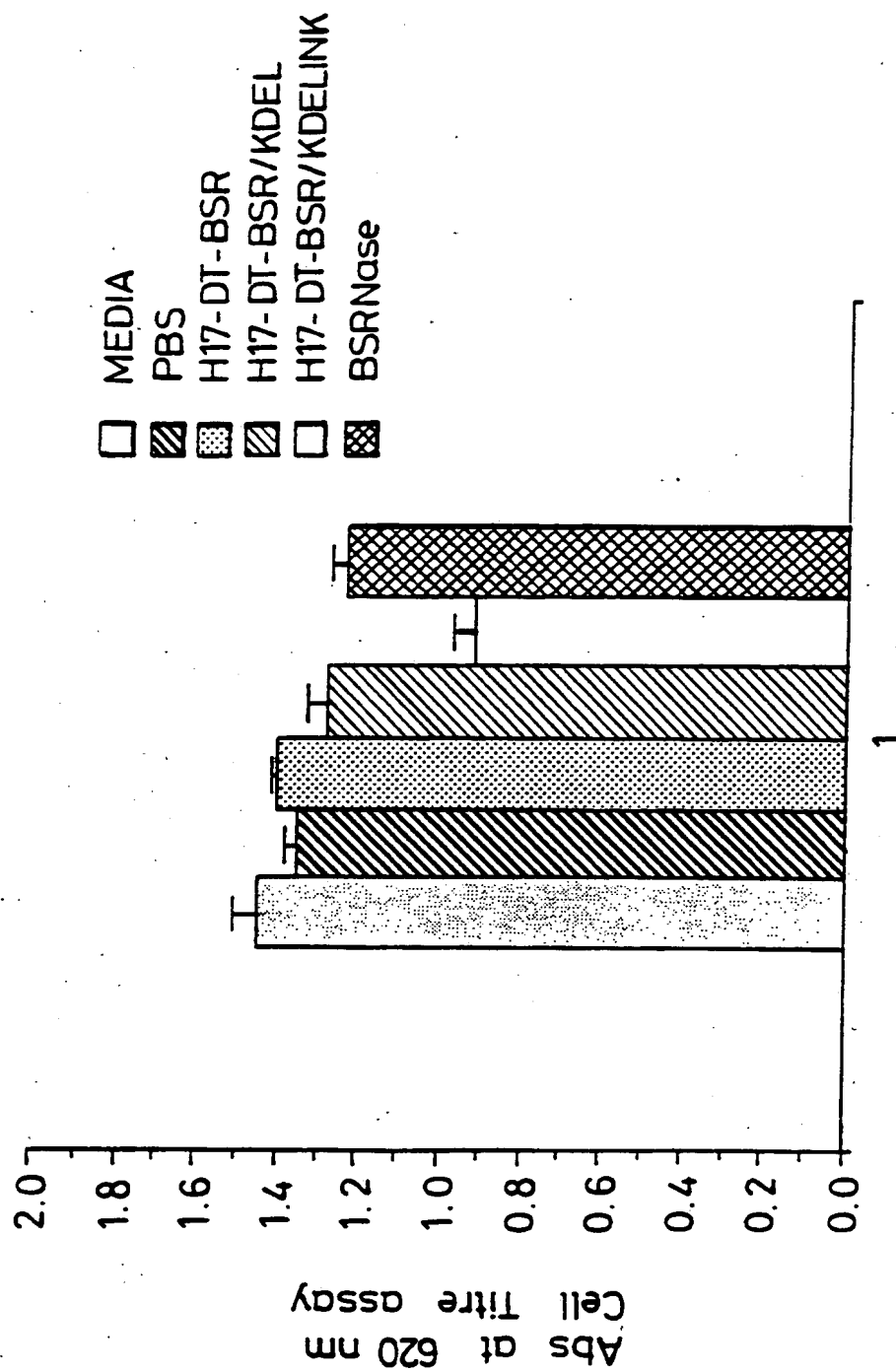


Fig. 27

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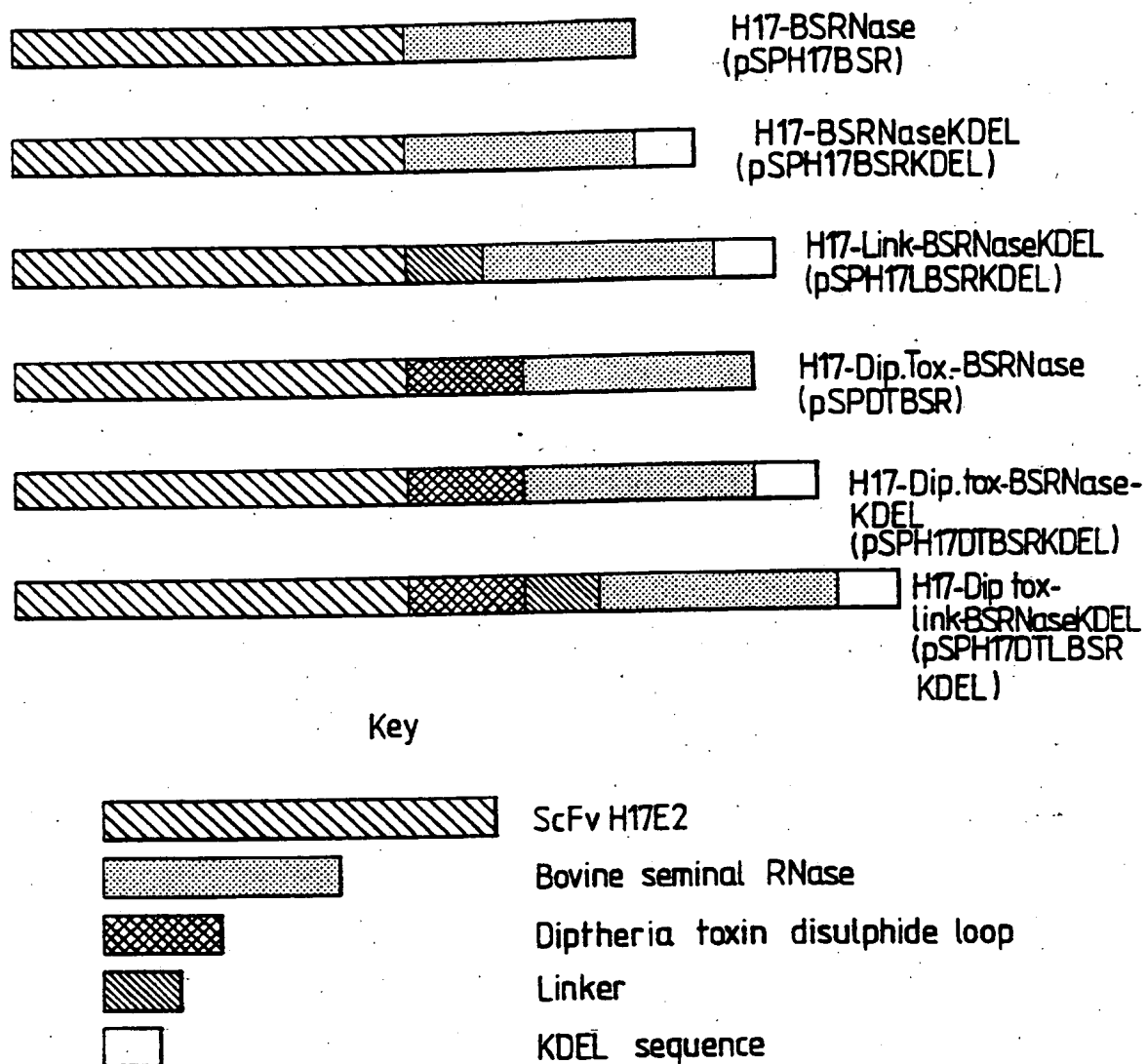


Fig. 28

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ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACC
AGCGATGGCCCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCC
TGTCATCACATGCACTGTCTCAGGGTTCTCATTAACCAGTTATGGTGTAAAGCTGG
GTTCCGCCAGCCTCCAAGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGGAAGACGG
GAGCACAATTATCATTACGTCTCATATCCAGACTGAGCATCAACAAGGATAACT
CCAAGAGCCAAGTTTTCTTAAACTGAACAGTCTGCAAACTGATGACACAGCCACG
TACTACTGTGCCAAACCCCACTACGGTAGCAGCAACGTGGGGGCTATGGAATACTG
GGGTCAAGGAACCTCGGTACCGTCTCCTCAGGTGGAGGCGGTTTACGGCGGAGGTG
GCTCTGGCGGTGGCGGATCGGACATCGAGCTCACCCAGTCTCCAGCCTCCCTAACT
GCATCTGTGGGAGAACTGTCAACATCACCTGTGAGCAAGTGAAAATATTTACAG
TTATGTAGCATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGTTCCTGGTCTATA
ATGCAAAATCCTTAGCAGAGGGTGTGCCATCAAGGTTTCAAGTGGCAGTGGATCAGGC
ACACAGTTTTCTCTGAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAATTATTA
CTGTCAACATCATTATGTTAGTCCGTGGACGTTTGGTGGAGGCACCAAGCTCGAGA
TCAAGCGCTCTAGCCTCGAAGGTGGGTGCGCTGGTAATAGAGTCAGAAGATCAGTC
GGAAGCAGCCTGTCTTGGCGGTGGTCTCGACGTGAGATCAAGCGCAAGGAATCTGC
AGCTGCCAAGTTTCGAGCGGCAGCACATGGACTCTGGCAACTCCCCAGCAGCAGCT
CCAATACTGCAACCTGATGATGTGCTGCCGGAAGATGACCCAGGGGAAATGCAAG
CCAGTGAACACCTTTGTGCATGAGTCCCTGGCCGATGTTAAGGCCGTGTGCTCCCA
GAAGAAAGTCACTTGCAAGAATGGGCAGACCAACTGCTACCAGAGCAAATCCACCA
TGCGCATCACAGACTGCCGCGAGACTGGCAGCTCCAAGTACCCCAACTGCGCCTAC
AAGACCACCCAGGTGGAGAAACACATCATAGTGGCTTGTGGCGGTAAACCGTCCGT
GCCAGTCCACTTCGATGCTTCAGTGTAG

FIGURE 29

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ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTA CTGCTGCCCCAACC
AGCGATGGCCCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCC
TGTCATCACATGCACTGTCTCAGGGTTCTCATTAACCAGTTATGGTGTAAAGCTGG
GTTCCGCCAGCCTCCAAGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGGAAGACGG
GAGCACA AATTATCATTACGTCTCATATCCAGACTGAGCATCAACAAGGATAACT
CCAAGAGCCAAGTTTTCTTAA AACTGAACAGTCTGCAAACTGATGACACAGCCACG
TACTACTGTGCCAAACCCCACTACGGTAGCAGCAACGTGGGGGCTATGGAATACTG
GGGTCAAGGAACCTCGGTCAACGTCTCCTCAGGTGGAGGCGGTT CAGGCGGAGGTG
GCTCTGGCGGTGGCGGATCGGACATCGAGCTACCCAGTCTCCAGCCTCCCTAACT
GCATCTGTGGGAGAACTGTCACCATCACCTGTGAGCAAGTGAAAATATTTACAG
TTATGTAGCATGGTATCAGCAGAAACAGGGAAGATCTCCTCAGTTCCTGGTCTATA
ATGCAAAATCCTTAGCAGAGGGTGTGCCATCAAGGTT CAGTGGCAGTGGATCAGGC
ACACAGTTTTCTCTGAAGATCAACAGCCTGCAGCCTGAAAATTTTGGGAATTATTA
CTGTCAACATCATTATGTTAGTCCGTGGACGTT CGGTGGAGGCACCAAGCTCGAGA
TCAAGCGCTCTAGCCTCGAAGGTGGGTGCGCTGGTAATAGAGTCAGAAGATCAGTC
GGAAGCAGCCTGTCTTGCGGTGGTCTCGACGTGAGATCAAGCGCAAGGAATCTGC
AGCTGCCAAGTTCGAGCGGCAGCACATGGACTCTGGCAACTCCCCAGCAGCAGCT
CCA ACTACTGCAACCTGATGATGTGCTGCCGGAAGATGACCCAGGGGAAATGCAAG
CCAGTGAACACCTTTGTGCATGAGTCCCTGGCCGATGTTAAGGCCGTGTGCTCCCA
GAAGAAAGTCACTTGCAAGAATGGGCAGACCAACTGCTACCAGAGCAAATCCACCA
TGCGCATCACAGACTGCCGCGAGACTGGCAGCTCCAAGTACCCCAACTGCGCCTAC
AAGACCACCCAGGTGGAGAAACATCATAGTGGCTTGTGGCGGTAAACCGTCCGT
GCCAGTCCACTTCGATGCTTCAGTGAAGGACGAACTGTAA

FIGURE 30

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ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCCAACC
AGCGATGGCCCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCC
TGTCATCACATGCACTGTCTCAGGGTTCTCATTAACCAGTTATGGTGTAAGCTGG
GTTCCGCCAGCCTCCAAGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGGAAGACGG
GAGCACAAATTATCATTCACGTCTCATATCCAGACTGAGCATCAACAAGGATAACT
CCAAGAGCCAAGTTTTCTTAAACTGAACAGTCTGCAAAGTATGACACAGCCACG
TACTACTGTGCCAAACCCCACTACGGTAGCAGCAACGTGGGGGCTATGGAATACTG
GGGTCAAGGAACCTCGGTACCGTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTG
GCTCTGGCGGTGGCGGATCGGACATCGAGCTCACCCAGTCTCCAGCCTCCCTAACT
GCATCTGTGGGAGAACTGTCAACCATCACCTGTGAGCAAGTGAATAATTTACAG
TTATGTAGCATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGTTCCTGGTCTATA
ATGCAAAATCCTTAGCAGAGGGTGTGCCATCAAGGTTTCAGTGGCAGTGGATCAGGC
ACACAGTTTTCTCTGAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAATTATTA
CTGTCAACATCATTATGTTAGTCCGTGGACGTTTCGGTGGAGGCACCAAGCTCGAGA
TCAAGCGCTCTAGCCTCGAAGGTGGGTGCGCTGGTAATAGAGTCAGAAGATCAGTC
GGAAGCAGCCTGTCTTGCGGTGGTCTCGACGTGAGATCAAGGCACCTGCTGCCTC
CCCGGCAGACGCTAAGGAATCTGCAGCTGCCAAGTTCGAGCGGCAGCACATGGACT
CTGGCAACTCCCCCAGCAGCAGCTCCAACCTACTGCAACCTGATGATGTGCTGCCGG
AAGATGACCCAGGGGAAATGCAAGCCAGTGAACACCTTTGTGCATGAGTCCCTGGC
CGATGTTAAGGCCGTGTGCTCCCAGAAGAAAGTCACTTGCAAGAATGGGCAGACCA
ACTGCTACCAGAGCAAATCCACCATGCGCATCACAGACTGCCGCGAGACTGGCAGC
TCCAAGTACCCCAACTGCGCCTACAAGACCACCCAGGTGGAGAAACACATCATAGT
GGCTTGTGGCGGTAAACCGTCCGTGCCAGTCCACTTCGATGCTTCAGTGAAGGACG
AACTGTAA

FIGURE 31

SUBSTITUTE SHEET

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ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCCAACC
AGCGATGGCCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCC
TGTCCATCACATGCACTGTCTCAGGGTTCTCATTAACCAGTTATGGTGTAAGCTGG
GTTCCGCCAGCCTCCAAGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGGAAGACGG
GAGCACAAATTATCATTACAGTCTCATATCCAGACTGAGCATCAACAAGGATAACT
CCAAGAGCCAAGTTTTCTTAAAACTGAACAGTCTGCAAACCTGATGACACAGCCACG
TACTACTGTGCCAAACCCCACTACGGTAGCAGCAACGTGGGGGCTATGGAATACTG
GGGTCAAGGAACCTCGGTACCGTCTCCTCAGGTGGAGGCGGTTCAGGCGGAGGTG
GCTCTGGCGGTGGCGGATCGGACATCGAGCTCACCCAGTCTCCAGCCTCCCTAACT
GCATCTGTGGGAGAACTGTCACCATCACCTGTGCGAGCAAGTGAAAATATTTACAG
TTATGTAGCATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGTTCCTGGTCTATA
ATGCAAAATCCTTAGCAGAGGGTGTGCCATCAAGGTTCAAGTGGCAGTGGATCAGGC
ACACAGTTTTCTCTGAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAATTATTA
CTGTCAACATCATTATGTTAGTCCGTGGACGTTCCGGTGGAGGCACCAAGCTCGAGA
TCAAGGCACCTGCTGCCTCCCCGGCAGACGCTAAGGAATCTGCAGCTGCCAAGTTC
GAGCGGCAGCACATGGACTCTGGCAACTCCCCCAGCAGCAGCTCCAACTACTGCAA
CCTGATGATGTGCTGCCGGAAGATGACCCAGGGGAAATGCAAGCCAGTGAACACCT
TTGTGCATGAGTCCCTGGCCGATGTTAAGGCCGTGTGCTCCCAGAAGAAAGTCACT
TGCAAGAATGGGCAGACCAACTGCTACCAGAGCAAATCCACCATGCGCATCACAGA
CTGCCGCGAGACTGGCAGCTCCAAGTACCCCAACTGCGCCTACAAGACCACCCAGG
TGGAGAAACACATCATAGTGGCTTGTGGCGGTAAACCGTCCGTGCCAGTCCACTTC
GATGCTTCAGTGAAGGACGAACTGTAA

FIGURE 32

SUBSTITUTE SHEET

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ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTA CTGCTGCCCAACC
AGCGATGGCCCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCC
TGTC CATCACATGCACTGTCTCAGGGTTCTCATTAACCAGTTATGGTGTAAGCTGG
GTTCCGCCAGCCTCCAAGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGGAAGACGG
GAGCACA AATTATCATTACAGTCTCATATCCAGACTGAGCATCAACAAGGATAACT
CCAAGAGCCAAGTTTTCTTAA AACTGAACAGTCTGCAA AACTGATGACACAGCCACG
TACTACTGTGCCAAACCCCACTACGGTAGCAGCAACGTGGGGGCTATGGAATACTG
GGGTCAAGGAACCTCGGTCACCGTCTCCTCAGGTGGAGGCGGTT CAGGCGGAGGTG
GCTCTGGCGGTGGCGGATCGGACATCGAGCTCACCCAGTCTCCAGCCTCCCTAACT
GCATCTGTGGGAGAAACTGTCACCATCACCTGTGAGCAAGTGAAAATATTTACAG
TTATGTAGCATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGTTCCTGGTCTATA
ATGCAAAATCCTTAGCAGAGGGTGTGCCATCAAGGTT CAGTGGCAGTGGATCAGGC
ACACAGTTTTCTCTGAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAATTATTA
CTGTCAACATCATTATGTTAGTCCGTGGACGTT CGGTGGAGGCACCAAGCTCGAGA
TCAAGCGCAAGGAATCTGCAGCTGCCAAGTTCGAGCGGCAGCACATGGACTCTGGC
AACTCCCCCAGCAGCAGCTCCAACTACTGCAACCTGATGATGTGCTGCCGGAAGAT
GACCCAGGGGAAATGCAAGCCAGTGAACACCTTTGTGCATGAGTCCCTGGCCGATG
TTAAGGCCGTGTGCTCCCAGAAGAAAGTCACTTGCAAGAATGGGCAGACCAACTGC
TACCAGAGCAAATCCACCATGCGCATCACAGACTGCCGCGAGACTGGCAGCTCCAA
GTACCCCAACTGCGCCTACAAGACCACCCAGGTGGAGAAACACATCATAGTGGCTT
GTGGCGGTAAACCGTCCGTGCCAGTCCACTTCGATGCTTCAGTGAAGGACGAAC TG
TAA

FIGURE 33

SUBSTITUTE SHEET

Elution of pRAS111 and pRAS112 proteins
from NP-Sepharose with 50 mM Glycine-
HCl pH2.2

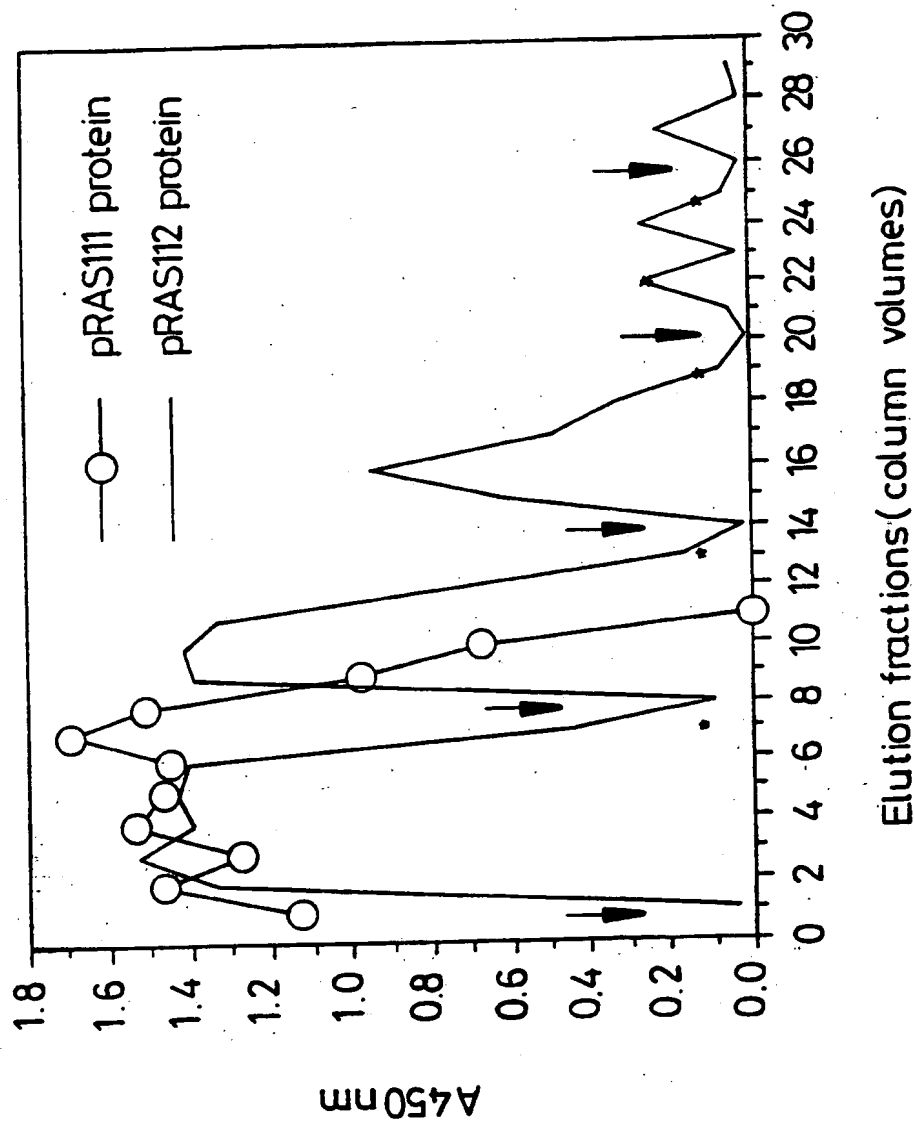


Fig. 34

INTERNATIONAL SEARCH REPORT

Inter. nat. Application No
PCT/GB 94/00087

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CELL BIOPHYSICS vol. 21, 1992 pages 121 - 138 S.M. RYBAK ET AL. 'RATIONAL IMMUNOTHERAPY WITH RIBONUCLEASE CHIMERAS'	1-5, 12, 21, 22, 24, 34-36, 41, 44
Y	see the whole document	23
X	WO, A, 91 16069 (THE UNITED STATES OF AMERICA) 31 October 1991 cited in the application	1-5, 12, 21, 22, 24, 34, 41, 44
Y	see abstract see page 7, line 27 - page 9, line 13 see page 10, line 28 - page 11, line 8 see page 21, line 22 - page 22, line 1; claims	23

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *Z* document member of the same patent family

Date of the actual completion of the international search

6 May 1994

Date of mailing of the international search report

6 May 1994

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Hoff, P

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 94/00087

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOBIOLOGY vol. 183, no. 3-4 , 1991 page 153 H. WEICHERT ET AL. 'COMPARISON OF IMMUNOCONJUGATES CONSISTING OF MONOCLONAL ANTIBODIES AND GLUCOSEOXIDASE OR RIBONUCLEASE T1' see the whole document ---	1-3, 12, 19, 22, 24, 25, 33, 34, 37-39, 41-44, 46
Y	---	23
Y	CANCER RESEARCH vol. 52, no. 12 , 1992 pages 3402 - 3408 T. YOKOTA ET AL. 'RAPID TUMOR PENETRATION OF A SINGLE-CHAIN FV AND COMPARISON WITH OTHER IMMUNOGLOBULIN FORMS' cited in the application see the whole document ---	23
Y	JOURNAL OF THE NATIONAL CANCER INSTITUTE vol. 82, no. 14 , 1990 pages 1191 - 1197 D. COLCHER ET AL. 'IN VIVO TUMOR TARGETING OF A RECOMBINANT SINGLE-CHAIN ANTIGEN-BINDING PROTEIN' cited in the application ---	23
X	EP,A,0 251 494 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 7 January 1988 see abstract. see page 4, line 30 - page 5, line 20 see page 6, line 38 - page 7, line 41; claims; example 2 ---	12, 19, 20, 22, 24, 25, 33, 37-39, 41-44
X	EP,A,0 496 074 (MINISTRO DELL'UNIVERSITA'E DELLA RICERCA SCIENTIFICA E TECNOLOGICA) 29 July 1992 see the whole document ---	12, 19, 22, 24, 25, 33, 37-39, 41 45
A	---	25-27, 46
X	EP,A,0 479 600 (UNILEVER) 8 April 1992 see abstract see column 3, line 17 - line 57 see column 5, line 7 - line 11; claims ---	25-27, 46
X	EP,A,0 140 728 (SANOPI) 8 May 1985 see abstract; claims ---	25-27, 46

	-/-	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 94/00087

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 263 046 (IMMUNOTECH) 6 April 1988	13,14, 16-18, 22,24,42
A	see abstract see page 3, line 20 - page 4, line 48; claims; examples 2-4 ---	25-28
A	CELL vol. 39, no. 3, 1984 pages 499 - 509 D. KALDERON ET AL. 'A SHORT AMINO ACID SEQUENCE ABLE TO SPECIFY NUCLEAR LOCATION' cited in the application see the whole document ----	9,10
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 265, no. 35, 1990 pages 21889 - 21895 A.F. WORRALL ET AL. 'THE CHEMICAL SYNTHESIS OF A GENE CODING FOR BOVINE PANCREATIC DNASE I AND ITS CLONING AND EXPRESSION IN ESCHERICHIA COLI' cited in the application ----	6-8,11, 31,32
A	WO,A,91 11201 (IMPERIAL CANCER RESEARCH TECHNOLOGY LIMITED) 8 August 1991 cited in the application see abstract see page 10, line 17 - line 24 see page 31, line 19 - page 32, line 8; claims -----	1-47

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB94/00087

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 42-47 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 94/00087

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO-A-9111201	08-08-91	EP-A- 0512002 GB-A, B 2256198 JP-T- 5503933	11-11-92 02-12-92 24-06-93